TARGETING AN IMMUNORECESSIVE TUMOR EPITOPE FOR ADOPTIVE IMMUNOTHERAPY OF ADVANCED-STAGE BRAIN TUMORS INDUCED BY SV40 T ANTIGEN

A Thesis in
Microbiology and Immunology

by

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The potential to recruit tumor-specific CD8+ T lymphocytes (T-CD8 cells) for the control of cancer has been investigated intensively for many years but has resulted in only limited success in human clinical trials. Such lack of translational success might be due in part to the use of particular animal models involving transplantable tumors, which fail to recapitulate natural cancer. This thesis project makes use of line SV11 mice, which express the oncoprotein SV40 large T antigen (Tag) as a transgene, in order to study the tumor-specific T-CD8 cell response in mice that develop spontaneously-arising choroid plexus tumors. Similar to some cancer patients, the T-CD8 cell response to the major tumor antigen in these mice - the Tag - is hindered by the development of T cell tolerance. Although T-CD8 cells specific for the three immunodominant Tag epitopes are deleted from the repertoire of SV11 mice during T cell development, a subset of Tag-specific T-CD8 cells, specific for the immunorecessive epitope V, survive in SV11 mice. This residual population of T-CD8 cells is composed of a mixture of both low and high avidity epitope V specific cells that can be recruited following a prime and boost immunization approach. To determine the effect of the tumor-bearing environment on higher avidity epitope V specific T-CD8 cells, the fate and anti-tumor potential of naïve T cell receptor transgenic T-CD8 cells (TCR-V cells) following adoptive transfer into SV11 mice with advanced tumors was investigated. TCR-V cells recognized the endogenous Tag post transfer into tumor-bearing SV11 mice, resulting in proliferation and acquisition of effector functions, but failed to accumulate in either the lymphoid tissues or at the tumor site. Specific immunization of SV11 mice following adoptive transfer of naïve TCR-V cells resulted in a modest expansion in the lymphoid organs, but failed to induce significant T cell accumulation at the tumor site.

Approaches aimed at augmenting epitope V targeted adoptive immunotherapy of SV11 mice bearing advanced stage tumors were thus investigated, and it was found that the cellular source of antigen, mode of antigen presentation, and timing of immunization played critical roles in eliciting the TCR-V cell response in vivo. The results demonstrate that the anti-tumor response of TCR-V T cells in tumor-bearing mice could be significantly enhanced by activation against both endogenous and exogenous sources of Tag. Specifically, strategically-timed cellular immunization targeted against the immunorecessive epitope promoted dramatic accumulation of functional TCR-V cells at the tumor site, which was associated with prolonged survival of SV11 mice. Tumor infiltration correlative with delayed tumor progression was also induced following the administration of an agonistic anti-CD40 mAb, suggesting that TCR-V cells were effectively triggered and primed against the
endogenous Tag following activation of resident antigen presenting cells, even in the presence of advanced tumors. Although both therapeutic approaches yielded comparable effects on the enhancement of SV11 survival, high levels of TCR-V cells did not persist long-term following either treatment regimen, and mice eventually succumbed to tumor burden. However, in contrast to either regimen administered individually, a combinatorial immunotherapeutic approach, consisting of both anti-CD40 pre-conditioning and immunization synergistically promoted long-term maintenance of TCR-V cells in the brain and extended SV11 survival. These data implied that adoptive T-CD8-mediated immunotherapy against spontaneously-arising tumors was optimized by enhanced sensitization against the endogenous tumor antigen and well-timed exogenous immunization.

It was also found that T-CD8 cell persistence at the tumor site did not always predict therapeutic efficacy, and that tumor burden at the onset of combinatorial immunotherapy may pose limitations on the capacity for TCR-V cells to control disease progression. Exploration of mechanisms contributing to the ultimate loss of tumor control despite long-term maintenance of TCR-V cells in SV11 mice post combinatorial immunotherapy, revealed that eventually the tumor environment in SV11 mice was characterized by the accumulation of anergic TCR-V cells and tumor infiltration by bone marrow-derived DCs expressing inhibitory receptors. These findings implicated TCR-V cell anergy - associated with persistent up-regulation of the inhibitory receptor PD-1 on tumor-infiltrating TCR-V cells compared with peripheral TCR-V cells - in the promotion of tumor escape from immune surveillance. However, the anergic phenotype was reversible, evidenced by the ability to restore function of non-responsive TCR-V cells ex vivo through blockade of a PD-1 receptor ligand. Furthermore, in spite of the failure of TCR-V cells from the initial transfer to exert continued anti-tumor function, SV11 tumors remained receptive to a second round of combinatorial therapy that included a fresh adoptive transfer of naïve TCR-V cells. These findings implicate critical checkpoints during tumor progression at which therapeutic intervention is necessary, namely (i.) optimizing initial enhancement of the priming event against endogenous tumor antigen, (ii.) providing appropriately-timed immunization to prolong the anti-tumor response, and (iii.) monitoring disease at late time points to overcome negative regulation of persisting cells. These studies demonstrate the potential for strategically-executed immunotherapeutic manipulations to dramatically augment a relatively weak anti-tumor response of immunorecessive epitope-specific T-CD8 cells in the face of advanced tumors, and imply that similar combinatorial therapeutic approaches toward cancer patients could yield promising results in clinical practice.
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<td>APC</td>
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<td>$\beta_2$-M</td>
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<td>cytotoxic T-lymphocyte-associated antigen 4</td>
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<tr>
<td>CY5</td>
<td>cytochrome-5</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DLI</td>
<td>donor leukocyte infusion</td>
</tr>
<tr>
<td>DLN</td>
<td>draining lymph node</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluoroisothiocyanate</td>
</tr>
<tr>
<td>Flu-HA</td>
<td>Influenza Hemaglutinin</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box protein 3</td>
</tr>
<tr>
<td>FR</td>
<td>folate receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Leukocyte functional antigen-1 (also known as CD11α/CD18)</td>
</tr>
<tr>
<td>LN</td>
<td>lymph node</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin’s Lymphoma</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PD-1</td>
<td>programmed cell death 1</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RAG</td>
<td>recombinase Activating Gene</td>
</tr>
<tr>
<td>RIP</td>
<td>rat insulin promoter</td>
</tr>
<tr>
<td>rHu</td>
<td>recombinant human</td>
</tr>
<tr>
<td>rVV</td>
<td>recombinant vaccinia virus</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAA</td>
<td>tumor-associated antigen</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>T-CD8</td>
<td>cytotoxic CD8+ T lymphocyte</td>
</tr>
<tr>
<td>TCR Tg</td>
<td>T Cell Receptor Transgenic</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDLN</td>
<td>tumor draining lymph node</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TIL</td>
<td>tumor infiltrating lymphocyte</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma mouse prostate</td>
</tr>
<tr>
<td>T-reg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>μm</td>
<td>micromolar</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

The completion of my thesis project would not have been possible without the support and guidance from people representing multiple facets of my life, including scientists, clinicians, Penn State University College of Medicine faculty, laboratory members, family, and close friends. I would first like to thank my mentor – Dr. Todd Schell – for his unwavering patience and dedication to seeing that I developed as a scientist. While Todd granted me much freedom and independence in the pursuit of my investigations, he continuously challenged me to seek the next level – in experimental design, writing technique, and oral presentation of my research. Likewise, every member of my thesis committee truly supported me - each in their own very individual way - and trained me how to formulate an answerable hypothesis and how to optimally design targeted experiments to test a proposed hypothesis. Much thanks to all of you – Dr. Sarah Bronson, Dr. M.J. Tevethia, Dr. Witold Rybka, Dr. Chris Norbury, and Dr. Shao-Cong Sun. I would also like to thank Dr. Courtney for welcoming me in my decision to pursue my graduate studies in Microbiology/Immunology, and for making an M.D./Ph.D. student feel right at home in the Micro Department. Furthermore, thank you to Tev – my biggest champion – who encouraged me tirelessly – and who taught me never to lose faith in myself.

Thank you to “Dr. Larry” Mylin from Messiah College for generation of the B6/V-only Tag cell line and to Dr. M.J. Tevethia for the creation of the TCR-V transgenic mouse line. I must acknowledge Melanie Epler for the preparation of tetramer reagent and Jeremy Haley for maintenance of the TCR-V transgenic mouse colony and for PCR typing of the SV11 mouse colony. I would like to acknowledge Dr. Ed Roy from University of Illinois for providing me with folate receptor antibody and Dr. Jelena Zinnanti, Byeong Lee, and Patti Zimmermann for their assistance with MRI imaging and data analysis. Thank you to the technicians in the animal facility and to the entire kitchen and administrative staff in the Micro Department. Thank you especially to Billie Burns for helping me to plan flights to scientific conferences, scheduling rooms for my committee meetings, and for keeping me on track for class registration and departmental deadlines.

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Those who know me understand that I could not dance, I could not breathe. I am thankful that during a rigorous academic schedule, I was still able to satiate my innate passion. But even more special for me was my opportunity to teach at the Hershey School of Dance. Thank you to all my girls – so full of energy and determination. Seeing you bring my choreography to life, and mature and develop as dancers truly filled me with new life – regardless of how beaten-down I may have been by school and science. I will miss you more than you know…

If you ask most graduate students, they will tell you that their parents are supportive of their studies, but do not really know what it is they actually “do” in lab. This is not the case with my family. Not only are my parents curious about my research, they really want to understand it. Neither of my parents have a scientific background. However, their continuous curiosity and questioning led to their own scientific development, whereby they describe my research project to other people with extreme accuracy and pride. After listening to my most recent investigations, they often asked pertinent questions and posed feasible experiments. Thank you for your steady faith in my potential to contribute to the field of medicine and research, and for being so patient at the other end of the phone line during calls of despair. I am lucky to have the two most awesome brothers: Anthony – a mechanical engineer who is not only one of the smartest guys I know, but also someone who was keenly aware of my hard work and sensitive to the stresses that often overwhelmed me; and Adam – a medical student at SUNY Buffalo who shares my love of immunology and who encourages and
reminds me that what I do is actually important. It was be so exciting to simultaneously experience clinical rotations with him starting in July, exchange stories of life on the wards as a med student, and to graduate together in 2009.

Most of all there is one person who has stood by my side every step of the way, and who promises to stay with me as I encounter whatever new challenges lie before me. Stuart – you are my boyfriend and my best friend, you have been there for me in so many ways – from running flow samples; to entering references for my comprehensive exam; to endlessly listening to my research triumphs and confusions during walks along the river in downtown Harrisburg; to understanding when an experiment went a lot longer than I had anticipated; to cooking delicious meals for me and bringing me lunch in lab just so we could see each other on a big analysis day; to constantly giving me strength and confidence that I could succeed. Achieving this goal would have been impossible without you, babe. I love you and am so very excited to marry you next year!

Finally, I give thanks to God - for granting me intelligence and the drive to work hard, and for surrounding me with so much support. I am so thankful for all the people who have guided me in pursuit of my doctoral degree. I am proud to take my place among so many talented and intelligent researchers. I pledge to use my knowledge to continue to inquire and learn, and I pray that my future investigations as a physician scientist will someday contribute to furthering new understanding in medicine.


This doctoral thesis project was supported by American Cancer Society Grant RSG-04-059-01-LIB.
I dedicate my thesis research project to my maternal grandparents –

Edward Francis Conners
and
Emma DeLuca Conners

- who both died of cancer long before I ever had an opportunity to meet them…
CHAPTER I

INTRODUCTION TO THESIS PROJECT

The appreciation that CD8+ T lymphocytes can venture beyond their policing role in the elimination of foreign pathogens to target tumors has provoked overwhelming enthusiasm for the development of alternate therapeutic strategies to treat cancer patients. The expression of cancer-associated antigens by tumors offers tempting opportunities to specifically target cancer cells in contrast to existing protocols that often indiscriminately sacrifice healthy cells in order to successfully destroy the tumor. Further encouragement for the promise of immunotherapy arose from the realization that cancer patients harbor tumor-reactive T cells in their endogenous repertoires, suggesting the potential for vaccination to tap such cells, by activating and inciting them to eliminate tumors. Unfortunately, this idealistic proposal has met with numerous challenges when applied to clinical situations. A particularly plaguing hurdle relates to the intrinsic nature of residual tumor-specific T cell populations detectable in cancer patients. As such cells are essentially “self-reactive,” they have likely been branded by tolerance and generally demonstrate poor responses to vaccination and limited capacity for anti-tumor effector function. Thus, the challenge for cancer immunotherapy charges biomedical and scientific explorations with unearthing treatment strategies that capitalize on the persistence of residual tumor-specific T cells in cancer patients, and subsequently applying this information for the design of therapeutic approaches that optimize the potential for tumor-specific T cells to prevail.

A. PROJECT RATIONALE AND SIGNIFICANCE

The focus of this thesis project was to determine the feasibility of recruiting T-CD8 cells specific for an immunorecessive tumor epitope (TCR-V cells) during immune surveillance of cancer. These studies made use of a mouse model in which expression of the oncogenic protein SV40 large tumor antigen (Tag) as a transgene results in the development of choroid plexus tumors (SV11 mice). Expression of Tag as a self-protein also leads to the inactivation of T-CD8 cells specific for dominant Tag epitopes, which ultimately leads to a crippled immune response to tumor. The detection cells in the endogenous repertoire of tumor-bearing mice of a residual population of T-CD8, that recognizes an immunorecessive epitope of Tag, offers the potential to recruit these cells for control of tumor burden. The significance of this project is demonstrated by the realistic setting in which an effective immune response must be mounted.
against a tumor that arises from within the host in the face of mechanisms that compromise the T cell response – a scenario observed in human cancer. By targeting a population of T-CD8 cells that escape tolerance to a tumor-expressing self antigen, it was possible to investigate the prospect of harnessing such tumor-reactive T-CD8 cells for the control of spontaneously developing cancer.

B. **HYPOTHESIS:** Within the context of a spontaneous tumor, immunotherapeutic strategies that target T-CD8 cells specific for an immunorecessive epitope, can be recruited for the control of cancer progression.

C. The objectives of this study were accomplished by exploring the following **SPECIFIC AIMS:**

**SPECIFIC AIM I:** To monitor the effect of endogenous Tag on the fate of immunorecessive Tag epitope V-specific T-CD8 cells (TCR-V cells) within the tumor-bearing host environment of SV11 mice, and the potential for TCR-V cells to control tumor progression.

Following adoptive transfer into SV11 mice, TCR-V cells were monitored for activation, proliferation, acquisition of effector function, tumor infiltration, *in vivo* persistence, and response to tumor growth. The response of TCR-V cells to the endogenous Tag as well to Tag-V specific cellular immunization was explored.

**SPECIFIC AIM II:** To explore potential strategies for enhancing the *in vivo* TCR-V cell-mediated anti-tumor effect, specifically approaches that promote persistence of TCR-V cells at the tumor site of SV11 mice.

The response of TCR-V cells to different immunotherapeutic regimens involving adoptive transfer into SV11 mice was investigated, specifically optimization of the *in vivo* activation against the endogenous Tag and variations in the kinetics and cell-type used for exogenous cellular immunization. The effects of immunotherapy were determined by the assessment of TCR-V cell expansion, functionality, tumor trafficking properties, persistence and effect on SV11 survival.
**SPECIFIC AIM III:** To investigate mechanisms of immune escape that permit tumor progression in SV11 mice despite initial efficacy of combinatorial immunotherapy and long-term persistence of TCR-V cells at the tumor site.

TCR-V cells that persisted in SV11 tumors following initially successful immunotherapy were isolated, and assessed for phenotype, proliferative potential and functionality in order to determine potential anergic tolerance. Additionally, SV11 tumors were analyzed for alterations potentially responsible for escape from TCR-V cell mediated immunotherapy.
I. Tumor Immunology: An Immunological Approach to Cancer Therapy

The earliest citation of treating a patient with cancer is recorded in Papyrus documents dated 1600 B.C., which describes the surgical excision of a tumor – an approach that remains the primary method of treating solid tumors today. Radiation therapy was introduced in 1896, and continues to play a role in treating certain tumor types, especially as adjuvant therapy. The dawn of chemotherapy in the 20th century introduced another potent weapon to the anti-cancer arsenal, and pharmacological induction of pancytopenia remains a common ingredient in many therapeutic protocols. The first recognition of the potential role of the immune system in cancer treatment is often accredited to Dr. William B. Coley, a 20th century New York City surgeon who observed spontaneous regression of some of his patients’ sarcomas – an event that coincided with resolution of an antecedent bacterial infection. Coley was the first physician to attempt to harness the power of the immune system to fight cancer (Coley, 1893). He deliberately infected cancer patients with live bacterial cultures, by injecting Streptococcus erysipelas directly into their growing tumor nodules. He subsequently formulated an “oncolysate vaccine” comprised of tumor cells admixed with bacteria – a treatment that ultimately prompted tumor killing and complete regression in some of his patients (Coley, 1894). Coupled with the success of effective microbial vaccines, these reports piqued interest in anti-tumor vaccination in the late 1800's and early 1900's, and a heightened energy towards developing cancer models for the study of tumor immunology was born. In the words of Paul Erlich - “If...it is possible to protect small laboratory animals in an easy and safe way against infectious and highly aggressive neoplastic specimens, then it will be possible to do the same for human patients” (Erlich, 1960).

It was not until 1976, upon the identification of interleukin-2 (IL-2) - known then as T cell growth factor - that it became possible to more fully understand how tumor recognition and rejection was orchestrated at the cellular and molecular level (Morgan et al., 1976). Based upon the observation that IL-2 drove in vivo expansion of T cells harboring specific immunological functions in both mice and man (Rosenberg et al., 1978a; Rosenberg et al., 1978b; Strausser and Rosenberg, 1978), IL-2 was administered to patients with solid tumors and shown to mediate anti-tumor immune responses which corresponded with regression of some metastatic
melanomas and kidney cancers (Atkins et al., 1999; Fyfe et al., 1995; Lotze et al., 1986; Rosenberg et al., 1985). Although the ultimate clinical impact was limited, these studies demonstrated the potential for immune manipulation to control advanced-stage bulky tumor burden, and established a benchmark that demanded investigations targeted toward understanding and enhancing immune cell-mediated cancer therapy (Shu et al., 1997).

In addition to their role in controlling intracellular pathogens, cytotoxic CD8+ T lymphocytes (T-CD8 cells) represent a crucial component of the adaptive immune system with the potential to seek out and destroy tumors. The induction of tumor immunity necessitates the fulfillment of several critical parameters: (i.) capture and processing of tumor antigens by antigen presenting cells (APCs); (ii.) selection, activation, and differentiation of antigen-specific T cells; and (iii.) homing of effector lymphocytes to the tumor site where recognition of tumor antigens leads to tumor elimination (Gilboa, 1999; Pardoll, 1998; Sogn, 1998) (Figure 1). T-CD8 cells are distinguished by their exquisite specificity for antigen based upon their expression of a clonotypically unique T cell receptor (TCR). The antigens recognized by tumor-specific T-CD8 cells consist of peptide fragments, derived from tumor proteins, which are bound within the major clefts of major histocompatibility complex (MHC) class I molecules on the tumor cell surface. In addition to cytolytic function, a number of other properties render T-CD8 cells attractive as mediators of anti-tumor immunity. The widespread expression of MHC class I molecules suggests that T-CD8 cells can be deployed against malignancies of diverse origin. Additionally, the T-CD8 cell is the ultimate effector cell shown to mediate rejection of solid tumors in pre-clinical animal models. Finally, T-CD8 cells continuously recirculate throughout the body to seek out antigen, a property useful for surveillance and treatment of systemic disease.

II. The CD8+ T Lymphocyte Response to Antigen

A. Anatomy of T Cell Activation

The generation of effector T-CD8 cells is characterized by tight regulation, and depends upon the antigen-driven differentiation of naïve T-CD8 cells (Bradley, 2003). This process is initiated by bone-marrow-derived APCs, such as dendritic cells (DCs), which acquire antigen within peripheral tissues and migrate to regional draining lymph nodes (DLNs) (Heath and
Effective destruction of tumors by antigen-specific CD8+ T cells is a multi-step process:

(a.) Tumor antigens must be present.
(b.) These antigens must be processed and presented by antigen-presenting cells (APCs) in the tumor draining lymph node.
(c.) Activated tumor specific T-CD8 cells must respond by proliferation.
(d.) The circulating T-CD8 cells must traffic to and infiltrate the tumor.
(e.) Once inside the tumor, the T-CD8 cells must be able to overcome local immune-suppressive molecules to recognize and kill target tumor cells.
(f.) Memory cells should be generated in order to provide lasting tumor protection.
FIGURE 1

T-CD8 Cells Respond to Tumors

Carbone, 2001b; Huang et al., 1994; Iyoda et al., 2002). This ideally situates DCs to interact with naïve T cells, which preferentially recirculate through secondary lymphoid tissue.

T lymphocytes develop in the thymus, and following maturation, selection, and export to the periphery, naïve T-CD8 cells traffic between blood and lymph, stopping intermittently in secondary lymphoid organs such as lymph node and spleen (Mackay et al., 1990). These tissues are specialized histologically to co-localize antigen, APCs, and naïve T cells, and peripheral lymph nodes are strategically located anatomically to facilitate collection and processing of antigen arriving in the lymphatic vasculature. Lymphocytes are carried into the lymph nodes via afferent lymphatic vessels, pass through the LN cortex and medulla, and exit via efferent lymphatic vessels. Such a flow pattern ensures that lymph-borne antigens will trickle through the densely clustered lymphocytes and APCs that constitute the inner node, increasing the probability that effective presentation will occur. The spleen has a similar organization, but lacks afferent lymphatic drainage, dedicating it to process blood-borne antigens. Naïve T cells utilize L-selectin and the chemokine receptor CCR7 to exit the blood and enter lymph nodes by binding to high endothelial venules (HEVs) – a mechanism not required for entry into the spleen (Girard and Springer, 1995; Mackay, 1993; Weninger et al., 2001). The chemokines that engage CCR7 include CCL19 – produced by stromal cells and mature DCs in the T cell zone of lymph nodes, and CCL21 – produced by endothelial cells of lymphatic vessels and HEVs and by stromal cells in the T cell zone (Gunn et al., 1998; Willimann et al., 1998). Dendritic cells that have acquired peripheral antigen also journey to the DLN guided by CCR7-mediated chemokine attraction (Dieu et al., 1998; Martín-Fontecha et al., 2003; Sallusto et al., 1998). Thus, both T-CD8 cells and DCs chemotactically traffic to DLNs in order to meet and initiate T cell activation.

B. Major Histocompatibility Complex (MHC) Restriction

i. Structure of the MHC

The response of the immune system to antigens derived from any cell type, including cancer cells involves a number of distinct receptor-ligand binding partners. T cells recognize peptide antigen fragments bound to the major histocompatibility complex (MHC), a cell surface glycoprotein – a process known as MHC restriction, which provides the basis for T cell antigen
discrimination (Germain and Margulies, 1993; Townsend and Bodmer, 1989; Yewdell and Bennink, 1992; Zinkernagel and Doherty, 1974). The MHC is encoded on chromosome 6 in humans and chromosome 17 in mice and includes at least 200 genes (Trowsdale, 1993). The classical MHC molecules, also referred to as human leukocyte antigens (HLA) in humans and H-2 genes in mice, play a vital role in the complex immunological dialogue that must occur between T cells and other cells of the body. At maturity, MHC molecules are anchored in the cell membrane, where they display short polypeptides to T cells. Recognition of the specific peptide-MHC complex by the T cell occurs via the unique T cell receptor (TCR). The presented peptides may be "self," (originating from a protein created by the organism itself), or foreign (originating from bacteria, viruses, etc.). The overarching design of the MHC-TCR interaction encourages T cells to ignore self-peptides while reacting appropriately to foreign peptides (1999; Delves and Roitt, 2000).

The major histocompatibility complex is divided into three regions - MHC class I, MHC class II, and MHC class III. The MHC class I encodes heterodimeric peptide binding proteins that include the genes for HLA-A, -B, and -C in humans and H2-K, -D, and -L in mice, as well as antigen processing molecules. The gene for \( \beta_2 \)-microglobulin (\( \beta_2 \)-m) - a component of the MHC class I complex - is encoded separately on chromosome 15 in humans and chromosome 2 in mice. The MHC class II includes genes for HLA-DR, -DP and -DQ in humans and H2-A, -O, and -E in mice, and encodes heterodimeric peptide binding proteins and proteins that modulate peptide loading onto MHC class II proteins in the lysosomal compartment. The MHC class III region encodes for other immune components, such as complement components and cytokines (Paul, 1999).

MHC class I molecules are expressed by most nucleated cells, whereas MHC class II molecules are expressed primarily by monocytes, macrophages, DCs and B cells. Each HLA molecule contains an antigen-binding groove, capable of binding a variety of peptide antigens. Individual peptides derived from a particular antigen are known as epitopes, or determinants. These peptides fit into the MHC groove with a unique configuration, and an antigen-MHC complex is presented to T cells in order to elicit antigen-specific T cell activity (Male, 2001). A functional heterodimeric MHC class I molecule consists of a single 45-kDa membrane anchored heavy chain - the \( \alpha \)-chain - which is folded into three subunits (\( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \)) non-covalently associated with a 11.5-kDa light chain \( \beta_2 \)-microglobulin (\( \beta_2 \)-m), and a peptide epitope containing
8-10 amino acid residues. Critical peptide amino acids responsible for stable contact with the MHC are termed anchor residues (Figure 2) (1999; Rammensee, 1998).

ii. **Mechanisms of MHC Presentation**

The assembly of MHC class I molecules with peptide is the culmination of a coordinated and regulated series of interactions with endoplasmic reticulum (ER)-resident chaperones and accessory molecules, and is characterized by the formation of the peptide-loading complex. The peptides are mainly generated in the cytosol by the proteasome (Goldberg and Rock, 1992; Rock et al., 1994; Townsend et al., 1988), which degrades intracellular proteins into small peptides that are then released into the cytosol. The peptides must be translocated from the cytosol into the endoplasmic reticulum (ER) lumen in order to meet with MHC class I molecules. The peptide translocation from the cytosol into the lumen of the ER is accomplished by the Transporter Associated with Antigen Processing (TAP). TAP is a member of the ABC transporter family and is a heterodimeric multimembrane-spanning polypeptide consisting of TAP1 and TAP2 subunits. The two subunits form a peptide-binding site and two ATP binding sites that face the lumen of the cytosol. TAP preferentially binds peptides with hydrophobic or basic carboxy terminal residues on the cytoplasmic side of the ER and translocates them under ATP consumption into the ER lumen (Monaco, 1992; Schumacher et al., 1994; Van Kaer et al., 1992; Yewdell et al., 1993). Within the ER lumen, peptides may be subject to further peptidase trimming by ERAP (Shastri et al., 2002), after which the final 8-10 amino acid residue peptides are loaded onto MHC class I molecules (Elliott et al., 1995; Snyder et al., 1994). The peptide-loading process involves several other molecules that form a large multimeric complex consisting of TAP, tapasin, calreticulin, calnexin, and ERP57. Once the peptide is successfully loaded onto the MHC class I molecule, it leaves the ER through the secretory pathway to reach the cell surface (Figure 3) (Yewdell and Bennink, 1989).

MHC class I molecules present peptides derived primarily from intracellular polypeptides to T-CD8 lymphocytes, whereas MHC class II molecules present antigens acquired extracellularly and interact with CD4+ T cells. The interaction between a TCR with peptide-MHC involves formation of a structure between the two cell interfaces, that is both complex and transient, termed an immunological synapse. The role of the synapse is to concentrate cell surface receptors (TCR, CD3, CD8/CD4, CD45, CD28) and integrins (LFA-1, I-CAM) allowing for optimal intracellular signaling to occur (Figure 4). The observation that the binding sites for
FIGURE 2

Structure of the Major Histocompatibility Complex (MHC)

A. MHC class I molecules are composed of an $\alpha$ chain which folds into three domains ($\alpha_1$, $\alpha_2$, and membrane-spanning $\alpha_3$), non-covalently bound to $\beta_2$-microglobulin, and a peptide binding cleft that accommodates peptide epitopes of 8-10 amino acid residues in length. Critical anchor residues in the peptide are responsible for stable binding of the epitope to the MHC.

B. MHC class II molecules consist of a non-covalent complex of an $\alpha$ chain and $\beta$ chain, both of which span the cell membrane, and a peptide binding cleft that accommodates peptide epitopes of at least 13 amino acid residues in length.

C. Engagement of MHC molecules and $\alpha$:$\beta$ T cell receptors (TCRs). The TCR interacts with both the MHC molecule and with the antigenic peptide. The complex is further stabilized by the interaction of co-receptors CD8 and CD4 for MHC class I and MHC class II, respectively.
FIGURE 2

Structure of the Major Histocompatibility Complex (MHC)

A. $\alpha_2 \alpha_1$
$\alpha_3$ $\beta$-microglobulin

MHC-Class I

B. $\beta_1 \alpha_1$
$\beta_2 \alpha_2$

MHC-Class II

C. MHC Engagement by T cell Receptor (TCR)

Reproduced from Nature 1098 Vol. 401 pp. 821-823
Intracellular proteins are presented via the MHC class I presentation pathway. Peptides derived from intracellular proteins are mainly generated in the cytosol by the proteasome, which degrades the protein into small peptides that are released into the cytosol. The peptides are translocated from the cytosol into the endoplasmic reticulum (ER) by the Transporter Associated with Antigen Processing (TAP) in order to meet with MHC class I molecules. Within the ER lumen, peptides may be subject to further peptidase trimming, after which the final 8-10 amino acid residue peptides are loaded onto MHC class I molecules. Once the peptide is loaded onto an MHC class I molecule, it leaves the ER through the secretory pathway to reach the cell surface.
FIGURE 3

MHC Class I Presentation Pathway

Reproduced from Yewdell, J.
Nature Reviews Immunology, 2003
Volume 3;12 p.952
Antigen-specific activation of T-CD8 cells occurs optimally in secondary lymphoid tissues where naive T cells traffic and follow chemokine gradients to interact with dendritic cells. Efficient presentation of peptide-MHC class I complexes to T-CD8 cells is achieved by mature dendritic cells that express high levels of MHC molecules, adhesion molecules and co-stimulatory molecules. The interaction between a TCR with peptide-MHC involves formation of a complex, transient structure between the two cell interfaces, termed an immunological synapse. The role of the synapse is to avidly concentrate T-CD8 cell surface receptors (TCR, CD3, CD8/CD4, CD45, CD28, 4-1BB) with DC ligands (MHC I, B7 molecules, 4-1BBL) allowing for optimal intracellular signaling to occur.
FIGURE 4

Activation of Naive T-CD8 Cells by Dendritic Cells

Reproduced from Walker, PR
Brain Res Brain Res Rev 2003, Volume 42;2 p. 101
CD4 and CD8 on MHC molecules were separate from the peptide binding domain of MHC molecules, and therefore from the site of interaction with the T cell receptor, suggested that a single MHC molecule could be bound simultaneously by both TCR and CD4 or CD8, increasing the overall avidity of the interaction (Figure 2c and Figure 4). Many reports have demonstrated that as few as 100 peptide-MHC complexes serially engage and trigger as many as 18,000 TCR (Davis, 1995; Germain, 1997; Valitutti et al., 1995). For T-CD8 cells, a brief period of time in the synapse (5-30 minutes) appears to be sufficient for initiation of signaling and liberation of lytic granules, which differs from CD4+ T cells, which require several hours of engagement before division and cytokine release are observed (Huppa and Davis, 2003; Lee et al., 2002).

iii. **Pathways of MHC Presentation**

a. **Endogenous Direct Presentation Pathway**

Direct presentation refers to the manner in which most endogenous cellular proteins are exposed to scrutiny on the surface of a cell, and utilizes antigen derived from intracellular proteins such as cellular peptides or infectious agents (Germain, 1999; Thery and Amigorena, 2001; Watts and Amigorena, 2000). Antigen presenting cells may directly express endogenous antigen that arises following infection or transfection of the cell, similar to the way other cells classically present endogenous proteins. Peptide fragments derived from intrinsic proteins are degraded in the proteasome, shuttled into the endoplasmic reticulum across TAP into the ER, where peptides are further trimmed to a length of 8-10 amino acids, loaded onto MHC class I molecules, and transported to the cell surface for extracellular display (Figure 3). The peptide-MHC complex can then be recognized by the TCR of a T-CD8 cell specific for the specific antigen epitope. In the context of a tumor cell, tumor antigen peptides bound to MHC class I molecules are recognized by a CD8+ T cell receptor specific for the tumor epitope (Antoniou et al., 2003; Falk et al., 1991a; Pamer and Cresswell, 1998; Shastri et al., 2002).

d. **Cross-Presentation Pathway**

Some cells are also able to capture antigens from an extra-cellular source and present these exogenous antigens through the classical MHC class I pathway (Heath et al., 2004). Cross presentation allows APCs such as DCs to induce T cells specific for antigens not expressed by the APC, whereby extracellular antigen (such as tumor material) is taken up and
shuttled into the MHC class I processing pathway for presentation to CD8+ T cells (Figure 5) (Bevan, 1976a; Brossart and Bevan, 1997; Chen et al., 2004a; Fonteneau et al., 2002; Guermonprez et al., 2002; Heath and Carbone, 2001b; Mellman and Steinman, 2001; Rock et al., 1993; Rodriguez et al., 1999; Staerz et al., 1987; Yewdell et al., 1999). The cross-presentation pathway incorporates elements of the endogenous and exogenous pathways, whereby internalized antigen that has been acquired by an APC can either be directly loaded onto MHC class I molecules via an endosomal-mediated mechanism or shuttled to the ER for processing and MHC class I loading (Bhardwaj, 2001; Fonteneau et al., 2001; Heath and Carbone, 2001b), ultimately resulting in presentation to T-CD8 cells (Albert et al., 1998b; Rovere et al., 1998). Although the mechanistic specifics underlying the union of these two established classical systems have not been completely elucidated, cross-presentation has been shown to involve endolysosomal compartmentalization (Lizée et al., 2003), proteins that participate in direct presentation (tapasin, calreticulin, ERp57), and may be TAP dependent or independent, according to the nature of the antigen (Cresswell et al., 1999; Houde et al., 2003). While the CD8α+CD11c+ DC is the primary cell type known for its ability to perform cross-presentation (Albert et al., 1998b; den Haan and Bevan, 2002; den Haan et al., 2000; Iyoda et al., 2002; Jung et al., 2002), other cells have also been shown to possess this capability in vitro, including B cells, endothelial cells, and macrophages (Ke and Kapp, 1996; Kovacsovics-Bankowski et al., 1993; Limmer et al., 2000; Rock et al., 1993; Savinov et al., 2003).

The first type of antigens reported to undergo cross-presentation were cellular antigens, in which following immunization of mice with allogeneic cells, minor histocompatibility antigen specific T-CD8 cells were induced. These T-CD8 cells were restricted by host MHC class I molecules and were contingent upon host APC capture of donor cellular antigens and processing through the MHC class I route of presentation (Bevan, 1976a, b). Numerous subsequent demonstrations of cellular antigen cross-presentation have been described (Carbone and Bevan, 1990; Gooding and Edwards, 1980; Huang et al., 1996; Huang et al., 1994; Kurts et al., 1996; Morgan et al., 1999b; Sigal et al., 1999). Additionally, soluble proteins, immune complexes, and intracellular bacteria and parasites may gain access to the cross-presentation pathway (reviewed in (Heath et al., 2004)). The mechanism by which an APC acquires captured antigens for cross-presentation may include phagocytosis, live cell nibbling (Harshyne et al., 2001), up-take of apoptotic cells (Albert et al., 1998b; Ferguson et al., 2002) or exosomes (Wolfers et al., 2001; Zitvogel et al., 1998), and heat shock proteins (Srivastava, 2002b). Additionally, LN resident DCs may acquire peripheral antigens via transfer of antigen.
FIGURE 5

Cross-presentation of Tumor Antigen to T-CD8 Cells

Cross presentation allows APCs such as DCs to induce T-CD8 cells specific for antigens not expressed by the APC, whereby extracellular antigen (such as tumor material) is taken up and shuttled into the MHC class I processing pathway for presentation to T-CD8 cells.
FIGURE 5

Cross-presentation of Tumor Antigen to T-CD8 Cells

Tumor Cell

Tumor Antigen

Antigen Presenting Cell (APC)

TCR

CD8

CD28

MHC I

MHC II

CD40

CD80/CD86
from tissue-derived migrating DCs, whereby resident DC populations may cross-present this antigen in order to induce immunity or tolerance (Carbone et al., 2004).

Cross-priming refers to cross-presentation that elicits T-CD8 cell activation, and responses to multiple types of antigens can be cross-primed, including those directed toward minor histocompatibility antigens, protein-coated cells that do not express MHC class I molecules, intracellular bacteria and parasites, DNA vaccination, viruses, self-antigens, and tumors. Cross-priming by a properly activated DC that has acquired exogenous antigen is demonstrated in multiple reports that describe the potent ability of activated DCs to elicit robust T cell responses. This capacity corresponds with expression of co-stimulatory molecules by the DC, including MHC class I and II molecules, CD80/CD86, OX40, CD27, and 4-1BB and cognate ligands on T cells – CD28, OX40L, CD70 (CD27L), and 4-1BBL (Banchereau et al., 2000; Lanzavecchia and Sallusto, 2001; Steinman et al., 2003). DCs may also cross-present antigen in the absence of co-stimulation, leading to death or anergy of antigen-specific T cells in a process known as cross-tolerance (Bansal-Pakala et al., 2001; Chen et al., 1999; Cooper et al., 2002; Diehl et al., 2002; Hendriks et al., 2000; Hernandez et al., 2001; Ohlen et al., 2002; Toes et al., 1998b).

e. **Exogenous Presentation Pathway**

The exogenous presentation pathway utilizes extracellular antigen by which APCs route exogenous proteins into endosomes, where they are processed into peptide fragments and loaded onto MHC class II molecules. The resultant antigen-MHC complex is presented on the APC cell surface, where it is recognized by an antigen specific TCR of a CD4+ helper T cell. Recognition induces the helper T cell to secrete cytokines that drive the cellular immune response (Germain, 1999; Roitt, 1997).
C. Antigen Presentation Orchestrates T-CD8 Priming

i. Dynamic Interactions Between T-CD8 Cells and APCs

Antigen presenting cells such as DCs are charged with a taxing assignment - the initiation of T cell activation. These cells represent the critical link between innate and adaptive immunity, instrumental in eliciting antigen-specific cellular immune responses (Hartgers et al., 2000). Dendritic cells function as sentinels poised in peripheral tissues, and are well equipped to capture antigens located throughout the body, process these antigens, and display large amounts of peptide-MHC complexes on their cell surface. DCs are extremely effective immunostimulatory APCs in which antigen uptake, processing, binding to MHC, and presentation to T cells occurs along the three afore-described pathways (Mellman and Steinman, 2001; Mellman et al., 1998). DCs must sample a generous fraction of the naïve T cell pool in order to physically engage potentially antigen-specific T cells and ultimately initiate activation and clonal expansion. In the presence of appropriate co-stimulatory molecules (CD80/CD86, CD40) DCs elicit potent antigen specific T cell mediated immune responses. DC maturation is critical to providing effective antigen presentation (Mailliard et al., 2000).

Immature DCs acquire tissue-derived antigen by endocytosis, pinocytosis, and phagocytosis (Fadok and Chimini, 2001; Henson et al., 2001; Hoffmann et al., 2001). Maturation signals (microbial or viral pathogens, cytokines, inflammatory mediators) induce DCs to downregulate antigen uptake and to upregulate expression of MHC, co-stimulatory molecules, and immunostimulatory cytokines such as IL1β, TNFα, and IL-12 (Albert et al., 1998a; Pulendran et al., 2001; Steinman et al., 1999; Watts and Amigorena, 2000). Mature DCs migrate from peripheral tissues to paracortical regions of regional lymph nodes, where antigen presentation to T cells occurs (Huang et al., 2000; McLellan et al., 2000).

The cellular dynamics underlying T cell/DC interaction in the LN have been observed in vivo using real-time 2-photon imaging in order to analyze DC/T cell behavior in their native environment. It was shown that DCs scan minimally 500 T cells/hour in absence of antigen, and while DCs can engage 10 or more T cells simultaneously over a duration of hours, the avidity of interaction influences the probability the T cell will be stably captured by a DC (Bousso and Robey, 2003; Delon et al., 2002; Stoll et al., 2002). While both immature and mature DCs may induce T cell division following only 2 hours of contact, it appears that 24 hours are required for
full programming of effector and memory responses (Kaech and Ahmed, 2001; Kaech et al., 2002; van Stipdonk et al., 2001). Successful T cell activation may depend upon proper maturation of the DC, facilitated by innate immunity triggers such as danger signals and TLR ligands or by adaptive immunity triggers such as cytokines and ligation of the CD40 receptor (Figure 6A).

ii. The Danger Milieu

The outcome of an antigen specific immune response is highly dependent upon the characteristics of the surrounding extracellular microenvironment. One hypothesis proposes that the immune system is capable of recognizing when a particular antigen derives from a threatening source (Fuchs and Matzinger, 1996; Matzinger, 1994, 1998). Antigen-directed immunologic activation is facilitated by immuno-stimulatory cytokines and inflammatory precursors associated with apoptotic and necrotic cell death, and some endogenous danger signals that have recently been discovered include heat-shock proteins, nucleotides, reactive oxygen intermediates, extracellular-matrix breakdown products, neuromediators, uric acid, and cytokines such as interferons (Gallucci and Matzinger, 2001; McLellan et al., 2000; Rovere et al., 1998). As “danger signals,” these mediators prime APCs for induction of potent antigen-specific cellular activity, characterized by DC maturation, costimulatory molecule up-regulation, and generation of antigen-specific T cell responses via the exogenous and cross-presentation pathways (Figures 6A and 6B) (Cella et al., 1999; Mailliard et al., 2000; Savill and Fadok, 2000; Somersan et al., 2001; Steinman et al., 1999). In the absence of inflammatory danger signals, immature DCs present antigen to T cells unaccompanied by appropriate cytokines and co-stimulatory molecules, potentially eliciting antigen-specific immunologic tolerance (Figure 6C). (Heath and Carbone, 2001b; Restifo, 2001a, b).

iii. APCs Determine Immunity Versus Tolerance

The ultimate fate of a naïve T-CD8 is determined by encounter with peptide antigens presented by MHC class I on the DC surface. While this event may induce T cell division, it does not necessarily predict a productive immune response. Thus, while a small proportion of T cells that recognize antigen on a resting DC may survive in draining lymph nodes (van Mierlo et al., 2002), most die prematurely in the absence of co-stimulation (Kaech and Ahmed, 2001; Kaech et al., 2002; van Stipdonk et al., 2001). Not only are DCs equipped to stimulate the
FIGURE 6

Potential Outcomes for T-CD8 Cell Interactions with an APC

The activated state of the APC may determine successful T-CD8 cell activation.
A. Under inflammatory conditions including viral infection, multiple signals, including danger signals (uric acid, heat shock proteins), Toll like receptor (TLR) stimulation, CD40 ligation, and inflammatory cytokines induce maturation of APC and successful T-CD8 cell activation.

B. For presentation of tumor antigens, the lack of multiple inflammatory conditions may fail to induce adequate APC maturation, thereby compromising T-CD8 cell activation.

C. For self-proteins, in the complete absence of inflammatory conditions, an APC maintains its immature phenotype, rendering T-CD8 cells tolerant towards the presented antigen.
FIGURE 6

Potential Outcomes for T-CD8 Cell Interactions with an APC

A. Cell and virus

B. Cell and tumour antigen

C. Cell and self proteins only

'Dangerous'
- Cell damage (e.g., uric acid)
- Innate immunity activated
- Toll-like receptors triggered
- Inflammation
- Cytokines
- CD40 signals
- Others

Weak
- No danger signals
- No CD40 signals
- Pro-inflammatory cytokines

Tolerizing
- None of these signals

T-CD8 Tolerance

Potential T-CD8 Activation

T-CD8 Activation

Adapted from Lake, R.A. & Robinson, B.W.S. Nature Reviews Cancer 2005, Volume 5;5, p.399
immune system against foreign antigen, they also constitutively present self-peptides such as those from the brain and peripheral organs, rendering DCs theoretically capable of activating high-avidity T-CD8 cells that recognize self antigen – a recipe for autoimmune disaster. However, the CD8α+ dendritic cell has emerged as the chief APC responsible for both initiation (cross-priming) of T cell responses as well as induction of T cell tolerance (cross-tolerance) (Belz et al., 2002; Heath and Carbone, 2001a, b; Liu et al., 2002b; Melief, 2003). Thus, DCs play a pivotal role in determining immunity versus tolerance, and are therefore often targets of immunomodulatory therapeutic modalities, both for boosting weak responses, and for dampening overly self-reactive responses (Ohashi and DeFranco, 2002).

The first theory proposed to explain how DCs regulate the decision between immunity and tolerance was put forth by Janeway (Janeway, 1992), and suggested that DCs possess pattern-recognition receptors that recognize conserved motifs on foreign pathogens, a concept subsequently supported by the discovery of Toll-like receptors (Medzhitov et al., 1997). The second theory proposes that destructive damage, due to inflammation, induces danger signals that alert the DCs to up-regulate co-stimulatory molecules and initiate an immune response. In the absence of danger, such as inflammation or adjuvant, DCs may continuously sample self-antigen for T cell presentation, but remain quiescent, and express low levels of co-stimulatory molecules (Steinman et al., 2003) without causing auto-reactivity (Kurts et al., 2001; Kurts et al., 1996; Lefrancois et al., 2000; Matzinger, 1994; Schmidt and Mescher, 1999). As a result, high-avidity recognition of self-antigens presented by DCs in the absence of co-stimulation leads to a brief period of proliferation and suboptimal development of effector cell function (Hernandez et al., 2001; Kearney et al., 1994).

In response to self-antigens, it has been proposed that the level of protein ingested by a DC determines whether T-CD8 cells are cross-primed or cross-tolerized, depending on the activation state of the DC (Kurts et al., 1997; Melief, 2003). One factor that may contribute to making this distinction involves the maturation status of the DC. DCs may be awoken from their resting state or “licensed” by the presence of inflammatory stimuli such as microbial LPS or cytokines (TNFα, GM-CSF), engagement of TLRs, or CD40 ligation. Such stimuli drive DCs to express activational markers, including members of the B7 family (CD80/CD86), to up-regulate levels of MHC class II and adhesion molecules, and to produce cytokines, such as IL-12. This mode of licensing matures the APC, thereby facilitating efficient activation of T lymphocytes, and it has been proposed that mature DCs initiate T cell activation, while quiescent DCs induce
tolerance (Figure 7) (Banchereau et al., 2000; Banchereau and Palucka, 2005; Banchereau and Steinman, 1998; Dhodapkar et al., 2001; Dhodapkar et al., 1999; Lambolez et al., 2002; Lanzavecchia, 1998). Thus, eclipsing of T cells that have engaged an immature DC appears to be the fate normally observed in T-CD8 cells responding to self-antigen, and tumor antigens frequently fall into this category, as they elicit low-level or improper DC activation (Figure 6) (Melief et al., 2000b; Qin et al., 1997).

iv. APC Licensing: The Role of CD40

Interaction between APCs and naïve T cells does not always lead to activation. A popular current model suggests that in order to achieve optimal T-CD8 cell priming, DCs must be in the activated, or “licensed” state. In the quiescent state, DCs express low levels of MHC and co-stimulatory molecules, and insufficiently generate T-CD8 cell responses. Thus, it has been proposed that the APC must be “licensed” by a CD4+ T cell in order to generate a full-fledged T-CD8 cell response (Guerder and Matzinger, 1992).

CD40 is a member of the tumor necrosis factor receptor (TNF-R) family, is expressed on DCs, B cells, macrophages, and activated T cells, and plays a pivotal role in orchestrating cell-mediated immune responses (Bourgeois et al., 2002; van Kooten and Banchereau, 1997). (reviewed in (Mackey et al., 1998a; Quezada et al., 2004; Toes et al., 1998a; van Kooten and Banchereau, 2000)). The cognate ligand for CD40 – CD40L (CD154) – is expressed on activated T cells (Klaus et al., 1997), B cells (Higuchi et al., 2002) and platelets (Danese et al., 2003; Henn et al., 1998), and has also been observed on vascular endothelial cells, peripheral blood monocytes, and peripheral blood monocytes during inflammation. Signaling events following ligation of CD40 on an APC involve trimeric clustering and intracellular recruitment of TNF-R associated factors (TRAFs), initiation of downstream signaling cascades, and activation of the NF-κB pathway, culminating in transcription of target genes (Banchereau and Steinman, 1998; Ouaaz et al., 2002; Pullen et al., 1998). The observed physiological read-out following CD40 stimulation is DC maturation - characterized by heightened antigen presentation, up-regulation of co-stimulatory molecules, cytokine (IL-12, IL-1, IL-6, TNFα) and chemokine production, and enhanced DC survival (Banchereau and Steinman, 1998; Frleta et al., 2003; Miga et al., 2001; Stout and Suttles, 1996). These maturational changes subsequently engender the DC with enhanced capacity to trigger T-CD8 cell activation, especially during the priming stage (Mackey et al., 1998a; Mackey et al., 1997). Indeed, it has been shown that
CD40 ligation is sufficient to drive the maturation of dendritic cells (Caux et al., 1994) and confer on them the ability to prime naïve T-CD8 cells (Schuurhuis et al., 2000).

Given multiple demonstrations of the capacity for CD40-triggered DCs to initiate T-CD8 cell responses, it was proposed that CD40 represented the in vivo “licensing signal,” whereby DCs present processed antigen to MHC class II restricted CD4+ T cells, inducing CD4+ T cells to up-regulate CD40L and subsequently engage CD40 expressed on the DC (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Smith et al., 2004). It was originally put forth that CD4+ T helper cells and T-CD8 killer cells recognized specific antigens simultaneously on the same APC, and that cytokine production by the CD4+ T cells enhanced activation of the T-CD8 cell. However, this theory was contingent upon the low probability of the two T cell types simultaneously engaging the same APC (Keene and Forman, 1982; Mitchison, 1982), and has faced re-evaluation, supported by the finding that T-CD8 cell responses do not always require T cell help (Buller et al., 1987).

It was ultimately shown by three groups that the CD40/CD154 interaction between a DC and a CD4+ T cell, respectively, could provide the alleged activating license. Bennett et al. (Bennett et al., 1998) and Schoenberger et al (Schoenberger et al., 1998) reported that mice lacking CD4+ helper T cells could not mount a T-CD8 cell response when injected with DCs expressing a T-CD8 cell-specific antigen. However, this failure was circumvented by injecting mice with an agonistic mAb that targeted CD40, essentially triggering the APC in vivo. Schoenberger’s group also showed that while in normal mice a CD4+ T cell-dependent response could be blocked with an antagonistic mAb to CD40L, this inhibition was alleviated with agonistic CD40 mAb (Schoenberger et al., 1998). In support of these findings, Bennett et al. showed the failure to elicit CD4+ T cell-dependent T-CD8 cell responses in mice lacking CD40 or CD40L (Bennett et al., 1998). Finally, Ridge et al. (Ridge et al., 1998) attempted to define the stimulus enabling APCs to activate T-CD8 by manipulating the APC in vivo. DCs could not activate a response unless they were first triggered by CD4+ T cells or anti CD40 mAb. Importantly, this interaction could be dissociated temporally, calling for a reassessment of the 3-cell liason theory, and implicating sequential interaction as sufficient for CD4+ T cell-dependent activation of T-CD8 by DCs.

These studies revealed the powerful effect of targeting CD40 for the enhancement of DC-mediated T cell responses, and shed light on novel avenues to manipulate DC activation in
Such findings implicated the potential to couple techniques such as administration of reagents that mimic CD40 ligation (recombinant CD40L or agonistic α-CD40 mAb) with vaccination and immunotherapy in order to augment T-CD8 responses to weak antigens such as tumor antigens.

**D. Properties of Efficiently Activated T-CD8 Cells**

The achievement of optimal T-CD8 cell expansion is thought to involve two sets of signals. Engagement of the TCR by the peptide-MHC complex satisfies the first signal - that is specific recognition of properly MHC-restricted antigen by the T cell. However, for effective T cell activation it is important that co-stimulatory B7 family member molecules on the APC (CD80/CD86) engage the CD28 receptor on naïve T cells (Kamath et al., 2000). This second signal complements the TCR-initiated signal, and results in robust clonal T-CD8 cell expansion and targeted attack against other cells expressing the same MHC-restricted antigen. Additionally, it has been shown that a third signal – such as the cytokine, IL-12, or adjuvant – may be necessary to promote effector function and memory in expanded T-CD8 cells, especially when antigen levels are limiting (Curtsinger et al., 2003; Curtsinger et al., 1999).

Once a naïve T-CD8 cell has received the proper stimulus following effective interaction between a TCR and a particular antigen-MHC complex, proliferation and clonal expansion ensue, with peak T cell frequencies generally observed between days 7 and 10 post-activation (Kaech et al., 2002; van Stipdonk et al., 2003; van Stipdonk et al., 2001). Full activation may also be facilitated by IFNγ and IL-12 secretion by CD4+ helper T cells. It has been shown that antigen specific T cells were initially activated in the DLN within two days post adoptive transfer and virus infection, during which 1-4 rounds of division occurred. These cells were not detected in the spleen until two days following expansion in the DLN, at which point extensive division had occurred (Coles et al., 2002). As T cells divide, they acquire various functions, including expression of IFNγ (Oehen and Brduscha-Riem, 1998). For optimal acquisition of effector status, it has been reported that satisfaction of several activation parameters must be achieved, including: (i.) sufficient strength of antigen stimulus and duration of T cell/APC engagement; (ii.) appropriate cell cycle status of the T cell being activated; and (iii.) a cytokine milieu supportive of T cell expansion and survival. Failure to meet these requirements may result in a program of incomplete activation and suboptimal stimulation, whereby cells demonstrate effector reactivity independent of cell division (Auphan-Anezin et al., 2003; Cao et al., 1995).
Following activation, T-CD8 cells alter their phenotype allowing their distinction from naïve T-CD8 cells. The surface phenotype of an activated T cell is characterized initially by expression of the earliest activation marker, CD69 (Ziegler et al., 1994), followed by up-regulation of CD44 (hyaluronic acid receptor); CD25 (IL-2 receptor) and CD45 (Walker et al., 1995; Waters et al., 2003). During the course of the primary immune response, activated T cells lose L-selectin and CCR7 expression, and acquire expression of adhesion molecules such as LFA-1 and VLA-4 that permit extravasation into non-lymphoid tissues. Trafficking of activated lymphocytes into peripheral sites is governed by interactions with endothelial receptors that are expressed on the vasculature of inflamed tissues (Sallusto et al., 1999; Sprent, 1994). The sum significance of these changes relates to the capacity of activated cells to proliferate, exit LNs and traffic to peripheral sites, extravasate through endothelium, and migrate through the extracellular matrix (ECM) towards target cells. Ultimately, effective priming initiates specific T-CD8 cell cytotoxic attack against other cells expressing the particular antigen, whereby T-CD8 cell killing of target cells may occur via either the perforin/granzyme-B pathway or death receptor-mediated apoptosis (Henkart, ; Krammer, 2000).

E. General Mechanisms of T Cell Migration

Lymphocytes do not follow random migratory pathways. Rather, they enter tissues from the bloodstream in a tightly regulated fashion, following a multi-step adhesion cascade (Butcher and Picker, 1996; Springer, 1994; von Andrian and Mackay, 2000). Blood-borne T cells initiate contact with endothelium, reflected by a decrease in velocity and rolling along the vessel wall. Engagement of chemokine receptors on rolling cells by chemokines presented on the endothelial surface causes conformational alterations of integrins, which facilitate firm arrest of the rolling cell against the vessel wall. Finally diapedesis across the endothelium achieves entry of the T cell into the target tissue. Once the cell has traversed the endothelial barrier, the next challenge posed is migration through the interstitial space – a process facilitated by interaction with stromal cells and ECM proteins, as well as by attraction along chemokine gradients. The mechanisms that govern each of the steps in trafficking include:
Poised to enter inflammatory sites, effector T cells downregulate secondary lymphoid organ homing receptors and express molecules such as P-selectin glycoprotein ligand (PSGL-1), α4β1 integrin, and LFA-1, which bind to E- and P-selectin, vascular cellular adhesion molecule-1 (VCAM-1), and intracellular adhesion molecule-1 (ICAM-1) respectively. Additionally, the chemokine receptors CCR2, CCR5, and CXCR3 are expressed to allow for homing into inflamed tissues. Effector T cells appear to demonstrate preference for homing to tissues of antigen priming, and recent evidence suggests that DCs within the specific microenvironments of secondary lymphoid organs imprint organ-specific patterns of homing molecules on T cells during priming (Mora et al., 2005; Mora and von Andrian, 2006).

### F. General T-CD8 Effector Functions

After binding to the peptide-MHC complex expressed on a target cell, an effector T-CD8 cell destroys the cell by perforating the cell membrane with enzymes (perforin/granzyme pathway) or by triggering a receptor-based (Fas/FasL or tumor necrosis factor receptor mediated) apoptotic pathway of self-destruction. Target recognition is extremely sensitive – a single peptide-MHC class I complex may trigger cytolysis by a high avidity effector T-CD8 cell (Sykulev et al., 1996). T-CD8 cells also employ non-cytolytic mechanisms against target cells, including production of interferon-gamma (IFNγ) - a cytokine with several direct and indirect cell-targeted properties (Qin et al., 2003).

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</tr>
<tr>
<td>Endothelium</td>
</tr>
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<td>CCL2, CCL5</td>
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<td>fractalkine</td>
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IFN\(\gamma\) is a member of the type II subset of IFN proteins, and plays multiple critical roles in protective immune responses (Bach et al., 1997; Boehm et al., 1997; Farrar and Schreiber, 1993; Schreiber and Farrar, 1993), many of which contribute significantly to control of tumors (Ikeda et al., 2002). In normal hosts, IFN\(\gamma\) production is limited to certain cell types, including natural killer (NK) cells that drive innate immunity and T lymphocytes that participate in the adaptive arm of the immune system. The induction of IFN\(\gamma\) from T cells occurs following antigen or mitogen stimulation of the T cell receptor, and the cytokine exerts its effects by interacting with a high affinity receptor expressed on virtually all cell types. The IFN\(\gamma\) receptor utilizes the JAK-STAT signal transduction pathway in order to regulate transcription of a multitude of IFN\(\gamma\)-inducible genes, contributory to the efficient execution of the immune response (Boehm et al., 1997; Farrar and Schreiber, 1993).

G. T-CD8 Cell Contraction

Eventually, following pathogen clearance, T cell numbers undergo a period of contraction (Badovinac et al., 2002), during which most of the clonally expanded cells die by apoptosis, leaving behind a small population of memory T cells (Dutton et al., 1998). It has been shown that the magnitude of the expansion phase reflects antigen dose and duration of antigen persistence. However, while these factors appear to determine the magnitude of T-CD8 cell expansion, this does not seem to be the case for contraction. The kinetics of contraction have been demonstrated to be independent of rate of antigen clearance, although the onset of contraction was different for primary and secondary responses. These data suggested that mechanisms of T cell contraction and homeostasis are programmed early after activation by a brief exposure to antigen, independent of antigen persistence (Badovinac et al., 2002). Some of the factors suggested to induce such programming include intrinsic factors such as susceptibility to apoptosis after initial activation and proliferation due to accumulated DNA damage, loss of mitochondrial potential, down-regulation of anti-apoptotic or up-regulation of pro-apoptotic proteins, and blocks in glucose use; and extrinsic factors including cytokine starvation, IFN\(\gamma\), and initiation of apoptosis through Fas/FasL interactions or tumor necrosis factor receptor (TNFR) induced signaling (Arch and Thompson, 1999; Badovinac et al., 2005; Derby et al., 2001; Grayson et al., 2000).
H. Establishment of T-CD8 Cell Memory/Survival

Following T cell contraction, a small residual population of memory T cells is retained in order respond with greater rapidity and amplitude should the host re-encounter challenge with the same pathogen. Generation of a memory population of T cells has been demonstrated to depend upon the initial strength of stimulation signal. While a period of short stimulation by either αCD3/αCD28 agonistic mAb triggering or antigen specific APC presentation led to T-CD8 cell proliferation in response to IL-2, these activated cells actually underwent an abortive cycle of proliferation and the resulting cells died by neglect due to the incapacity to proliferate in response to IL-7 or IL-15. However, prolonged stimulation enhanced cytokine responsiveness, effector function, and enhanced T-CD8 cell survival (Gett et al., 2003; van Stipdonk et al., 2003). It was subsequently shown that CD4+ T cell help was necessary for the generation and survival of memory T-CD8 cells (Bevan, 2004; Sun et al., 2004), and the importance of CD4+ T cells in the licensing of the DC prior to priming has been implicated in the achievement of functional memory populations (Smith et al., 2004).

Naïve and memory T cells differ in their requirements for expansion and survival. Whereas naïve T cells require antigen to expand and engagement of the proper MHC class I to survive, memory cells required only the correct MHC class I for expansion in the absence of antigen, and non-specific TCR-MHC interactions for survival (Tanchot et al., 1997a). In order to further determine how memory and naive T cells differ, large numbers of long-lived memory T-CD8 cells were generated and compared to naive cells expressing the same antigen-specific T cell receptor. Although both populations expressed similar levels of TCR and CD8, upon antigen stimulation in vitro, memory T cells down-regulated their TCR faster and more extensively and secreted IFNγ and IL-2 more rapidly than naive T cells. Memory cells were also larger, and when freshly isolated from mice, contained perforin and killed target cells without the need for restimulation. They further differed from naive cells in requiring IL-15 for proliferation and demonstrating a greater tendency to undergo apoptosis in vitro. Under steady-state in vivo conditions, memory cells appear to divide slowly (Bruno et al., 1996; Murali-Krishna et al., 1999). However, following antigen re-stimulation in vivo, they proliferated more rapidly than naive cells. These findings suggest that, unlike naïve T cells, memory T-CD8 cells are intrinsically programmed to rapidly express their effector functions in vivo without having to undergo clonal expansion and differentiation (Cho et al., 1999).
In terms of T-CD8 lymphocyte homeostasis, naïve and memory cells occupy separate niches. While the total number of T cells remains constant (2x10^7 CD44- cells/mouse), newly generated thymic migrants (10^6/day) do not replace memory cells. Rather, in order to make space for new cells, exponential decay of resident tolerant T cells occurs, avoiding competition for identical niches while preserving populations of previously generated memory cells. Additionally, large clones are eliminated at an accelerated rate, in order to maintain a diverse T cell repertoire (Tanchot and Rocha, 1995, 1997; Tanchot et al., 1997b).

Cytokines such as IL-7 and IL-15 are critical in promoting maintenance of the peripheral memory T cell compartment (Tuma and Pamer, 2002), compared to IL-2 inhibition of memory survival. The receptors for IL-15 and IL-2 share the IL-2Rβ chain (CD122), and memory T cells bear high levels of this receptor (Cho et al., 1999; Nelson and Willerford, 1998). An antibody to IL-2 was found to increase the number of proliferating cells in vivo, suggestive of a balancing act between IL-15 and IL-2 for the same receptor during memory cell homeostasis (Ku et al., 2000). Furthermore, in addition to IL-7 and IL-15, the relative affinity of the TCR, as determined by CD5 expression, contributes to the ability for memory cells to homeostatically proliferate and compete for survival (Kieper et al., 2004).

Memory T-CD8 cells have been divided into 2 general populations with unique capacities to partition between lymphoid and non-lymphoid compartments. Central memory cells express CCR7 and L-selectin, persist in lymphoid tissues, and possess high IL-2 responsive proliferative potential. Effector memory cells are CCR7^{low} and L-selectin^{low}, are maintained in peripheral tissues, and demonstrate elevated production of IFNγ (Sallusto et al., 2004; Sallusto et al., 1999). The generation of such distinct memory T-CD8 cell populations ensures that T-CD8 cells are readily available to respond in the tissues (effector memory cells) while a separate wave of T-CD8 cells undergoes clonal expansion in secondary lymphoid tissues (central memory cells), ultimately providing optimal protection, should the host face antigen re-challenge.
I. T-CD8 Cell Death

At least two different types of apoptotic T cell death are thought to occur – activation induced cell death (AICD) and activated T cell autonomous death (ACAD), and these pathways function through separate mechanisms according to the events that precipitate removal of the T cell (Hildeman et al., 2002b). T cell survival in the periphery is governed by a balance between endogenous pro-apoptotic and anti-apoptotic molecules, which function to regulate mitochondrial integrity (Zhu et al., 2004). For example, the level of the anti-apoptotic molecule bcl-2 is maintained in resting T cells by signaling through IL-7Rα (CD127), and functions to inhibit the activity of the pro-apoptotic molecule, Bim (Hildeman et al., 2002a; Schluns et al., 2000).

ACAD apoptosis is favored by single-dose exposure to foreign antigen, and plays a critical in vivo role in the removal of T cells at the conclusion of an immune response (Hildeman et al., 2002b). It is not driven by death receptors such as Fas/FasL interactions or tumor necrosis factor (TNF) receptors. ACAD is driven by Bim (protected by bcl-2 expression), is caspase dependent, and is sensitized by cytokine withdrawal. In contrast, AICD apoptosis is not responsible for ending the immune response to foreign antigen. Rather, it is favored by high doses of antigen or repetitive stimulation, and contributes to the control of self-reactive T cells in vivo and induction of tolerance. AICD is mediated by engagement of death receptors and is caspase dependent (Duke, 1992). Additionally, it demonstrates sensitivity to IL-2 and is not protected by expression of bcl-2. As an example of the separate control pathways governing these two modes of cell death, over-expression of bcl-2 prolongs the T cell response to foreign antigen, while T-CD8 cells may still be tolerized to self-antigen (Van Parijs et al., 1998).

III. Tolerance

A functional immune system is contingent upon the selection of T cells that express receptors restricted by major histocompatibility complexes and reactive towards foreign antigen, yet tolerant of self antigens. When properly executed, tolerance promotes the selection of T lymphocytes that are poised to attack and eliminate cells expressing foreign antigen, yet incapable of inducing autoimmunity. The main mechanism by which auto-reactive T cells are
purged from the potential repertoire occurs via clonal deletion in the thymus. Additionally, since not all self antigens are presented in the thymus, mechanisms exist in the periphery to eliminate self-reactive cells that may have escaped thymic scrutiny (Stockinger, 1999).

A. Central Tolerance

When bone marrow stem cells destined to become T lymphocytes enter the thymus, they express neither CD4 nor CD8 (double negative, or DN stage). These immature precursors accumulate in the thymic cortex, a region richly populated by specialized epithelial cells that express high levels of MHC molecules. It is here that immature T cells begin to rearrange their TCR, starting with the TCRβ chain. Eventually, both CD4 and CD8 are co-expressed (the double positive, or DP stage) and the TCRα chain is rearranged, ultimately creating thymocytes with TCRs capable of recognizing billions of unique determinants (Goldrath and Bevan, 1999). Evidence from fetal thymic organ cultures indicates that DP thymocytes that express TCRs with low, yet measurable avidity for a self-peptide complexes presented on cortical epithelial cells will be positively selected and permitted to develop into mature CD4+ or CD8+ T cells – a decision proposed to be dependent upon strength of the TCR signal (Watanabe et al., 2000). In contrast, a cortical DP thymocyte that expresses a TCR with no avidity for self-peptide-MHC will not survive and will die via apoptosis (Mondino et al., 1996; Sprent, 1995; Starr et al., 2003).

Thymocytes that survive positive selection migrate deeper into the medulla where they interact with bone marrow-derived APCs that express self-antigen (Gallegos and Bevan, 2004; Palmer, 2003). Intra-thymic expression of self-antigen increases the chance that immature T cells specific for the specific antigen will be purged, but the affinity and avidity of interaction between a single positive T cell and MHC class I-self peptide complex determines final selective fate (Hanahan, 1998; Jolicoeur et al., 1994; Starr et al., 2003). It is postulated that since a TCR that exhibits strong avidity for self-peptide/MHC portends future auto-reactivity, these thymocytes are subsequently deleted. However, a TCR that recognizes self-peptide/MHC, but binds with weak affinity will survive negative selection and will exit the thymus to seed secondary lymphoid organs. Models of repertoire development in the thymus have demonstrated that positively selected cells exiting the thymus express TCRs that display a strength interaction with self peptide/MHC that exceeds the threshold required for positive
selection and rescue from programmed cell death, yet below that which leads to deletion (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Liu et al., 1995).

The immune system is equipped with multiple levels of insurance to protect the body from auto-immunity. This arsenal includes the presence of the transcription factor AIRE (autoimmune regulator) – expressed by medullary epithelial cells in the thymus – which promotes the presentation of a portfolio of tissue-specific proteins not ordinarily expressed by these APCs (Anderson et al., 2002). Thymic expression of AIRE allows for a greater sampling of peripheral self-antigens during the process of selection, optimizing the elimination of potentially auto-reactive thymocytes. It has also been reported that antigen levels in the thymus play a significant role in negative selection (Oehen et al., 1994). The concentration of antigen required to induce full tolerance in the thymus has been shown to be on the order of $10^{-8}$ M – $10^{-9}$ M, while lower antigen concentrations elicit incomplete tolerance (Robertson et al., 1992; Stockinger and Hausmann, 1994). Thus, for antigens not expressed or expressed at negligible levels in the thymus, self-reactive T cells may escape tolerance, and thereby remain responsive to peripheral antigen either at normal or unphysiologically high concentrations (Sprent, 1995).

Ultimately, following thymic selection, the T lymphocyte repertoire exported to the periphery consists of high-affinity cells capable of recognizing poorly processed epitopes and epitopes not represented in the thymus, in addition to low-affinity cells that recognize well-processed self-peptide/MHC complexes (Liu et al., 1995; Morgan et al., 1998). As they circulate and patrol the body, naïve T cells require intermittent “tickling” by low-affinity interactions with self peptide-MHC to retain alertness, thereby protecting T cells against death by neglect and reaffirming T cell potential to respond to higher affinity interactions with foreign peptide (Goldrath and Bevan, 1999). Additionally, during their lifetime in the periphery, any residual, strongly auto-reactive cells may be further subject to antigen-specific tolerance.

**B. Peripheral Tolerance**

Although efficient thymic selection removes many self-reactive T cells, some antigens are developmentally regulated (expressed later in life) or expressed exclusively in a particular tissue. This precludes any opportunity to purge the repertoire prior to peripheral export, and such self-reactive T-CD8 cells may be capable of inducing auto-immunity (Kreuwel et al., 2001; Miller and Morahan, 1992). A disadvantage to the postponement of tolerance until after a self-
reactive T-CD8 cell reaches the periphery is that in contrast to thymocytes, which are deleted by a strong TCR signal without initiating cellular division, a potent TCR signal in mature T cells is programmed to drive proliferation. Thus, peripheral tolerance mechanisms must contend not only with the autoreactive T cell, but with its clonal progeny as well (Gett et al., 2003; Kishimoto and Sprent, 1997).

Multiple lines of protection are responsible for keeping auto-reactive cells at bay in the periphery, and several theories propose to explain the mechanisms that drive such T cell tolerance. As in central tolerance, high antigen concentration has been shown to dictate susceptibility to peripheral tolerance via deletion of T-CD8 cells specific for self-antigen (Kurts et al., 1999; Morgan et al., 1999a; Morgan et al., 1999b). Alternatively, T cell avidity has been proposed as the factor that governs whether a T-CD8 cell is deleted from the periphery (high-avidity) or rendered non-responsive (low-avidity) (Sandberg et al., 2000). Depending on the level, localization, timing, and chronic nature of antigen expression, induction of extra-thymic tolerance may take the form of ignorance, deletion, or anergic non-responsiveness (Aichele et al., 1995; Arnold et al., 1993; Redmond and Sherman, 2005; Rocha et al., 1995; Rocha and von Boehmer, 1991; Rocken and Shevach, 1996; Walker and Abbas, 2002; Zajac et al., 1998; Zinkernagel, 2000). By comparing the T cell repertoire that emerges in the presence or absence of self-antigen, T-CD8 cells that survive thymic and peripheral deletion - the tolerant repertoire - has been characterized by: (i.) diminished avidity for self antigen-MHC complexes; (ii.) elevated threshold of peptide required for triggering of effector function; and (iii.) reduced strength of signal transduction following self-antigen recognition. Additionally, if high-avidity self-reactive T-CD8 cells do escape to the periphery, they are generally available in significantly reduced numbers (de Visser et al., 2000; Hernandez et al., 2001; Hernandez et al., 2000; Murtaza et al., 2001; Nugent et al., 2000; Sandberg et al., 2000; Theobald et al., 1997).

i. Mechanisms of Peripheral Tolerance

a. Ignorance

Since naïve T cells are relatively confined to the blood and secondary lymphoid organs, they do not efficiently enter un-inflamed non-lymphoid tissues. Therefore, antigens expressed exclusively in non-lymphoid organs may be tolerated simply because naïve T cells do not have access to the tissues that express them. As a result, these T cells remain “ignorant” of such
antigens, and T cell responses against these antigens are undetectable (Alferink et al., 1998; Andre et al., 1996; Hoglund et al., 1999; Kurts et al., 1998a; Ohashi et al., 1991). Additionally, it has been proposed that T-CD8 cells may remain ignorant of tumors situated in remote anatomical locations, especially if levels of tumor antigen are insufficient (Kurts et al., 1999; Spiotto et al., 2002; Zinkernagel, 2002).

Some anatomical sites, such as the brain, gonads, and eye, are considered “immune privileged sites” because foreign antigens placed in these sites do not provoke an immune response – leading to tolerance instead. These organs maintain extremely stringent barriers to routine entry of naïve lymphocytes, as such restriction is critical for avoiding disruption of normal physiologic function induced by the destructive force of an immune response. The blood-brain-barrier (BBB) represents such a protected site, where tight junctions between endothelial cells of brain vasculature prohibit the access of lymphocytes to the central nervous system (Girard and Springer, 1995; Schlosshauer, 1993). Thus, for immune privileged sites, ignorance may play a critical role in maintaining tolerance (Ksander and Streilein, 1994).

b. **Deletion**

For newly encountered peripheral antigens that are not expressed in the thymus, it has been shown that in the absence of adjuvant or other inflammatory stimulus, naïve T cells undergo several rounds of abortive proliferation that ultimately results in their clonal elimination (Kearney et al., 1994; Kurts et al., 1997; Kyburz et al., 1993; Mamalaki et al., 1993; Matzinger, 1994; Rocha et al., 1995). The main apoptotic mechanisms responsible for tolerogenic T cell deletion are the mitochondrial (Bim/Bcl-2) and receptor-mediated (Fas/FasL-dependent) pathways (Ferguson and Griffith, 1997; Steinman et al., 2000). There is evidence to support that cell death of naïve self-reactive T cells is attributable to Bim-mediated apoptosis, while tolerogenic deletion of previously activated/memory T cells is critically dependent on IL-2 and is linked to Stat 5 induction of Fas/FasL interactions (Cohen et al., 1992; Davey et al., 2002; Kabelitz et al., 1993; Lenardo, 1991; Van Parijs et al., 1998; Watanabe-Fukunaga et al., 1992; Wesselborg et al., 1993). FasL-induced apoptosis has also been shown to be an important mechanism of cell death and tolerance in immune-privileged sites (Griffith et al., 1996). Interestingly, peripheral tolerance mediated by Fas-dependent deletion of antigen specific T cell clones may be orchestrated by other endogenous T-CD8 cells (Herndon et al., 2005). Thus, under steady-state conditions, the immune system recognizes that self-antigens are to be
tolerated, and utilizes self-reactive T cell deletion as a means of maintaining immune homeostasis.

c. Anergy

Another mechanism of preventing auto-reactivity orchestrated by T cell surveillance of peripheral tissues is the induction of non-responsiveness, or anergy. Anergy has been defined as “…a state of long-lasting, partial, or total unresponsiveness induced by partial activation” (Lechner et al., 2001), and has been attributed to uncoupling of antigen-driven TCR activation from down-stream signaling pathways (Macian et al., 2004). Anergy induction in vivo has also been defined as “adaptive tolerance” and has been proposed to be essential during chronic infection in order to control excessive expansion of antigen-specific T cells and minimize the activation of self-reactive lymphocytes (Schwartz, 2003). This phenomenon was initially observed in CD4+ T cells in vitro and was reported to stem from TCR ligation in the absence of co-stimulation (Jenkins and Schwartz, 1987). Anergy also affects T-CD8 cells, and has similarly been described as an active process that occurs as a result of TCR engagement without co-stimulation (Macian et al., 2002), although T-CD8 cell tolerogenic hyporesponsiveness and clonal deletion in vivo have also been reported to depend upon CD28/B7 interactions (Vacchio and Hodes, 2003).

Anergy appears to be dependent upon the continuous presence of antigen, as T-CD8 cells regain the ability to respond provided they are permitted to rest in an antigen-free environment (Rocha et al., 1993; Schwartz, 2003). The anergic state may be characterized by down-regulation of TCR, CD8, or co-stimulatory molecules - resulting in decreased T cell avidity and non-responsiveness to antigen stimulation (Hammerling et al., 1991; Schonrich et al., 1994; Singer and Abbas, 1994). Anergic cells may lose their capacity to proliferate or produce effector cytokines in response to antigen stimulation or may display impaired trafficking capability, resulting in accumulation within the LN where initial activation or tolerance was induced (Charles et al., 1999; Kearney et al., 1994; Lechner et al., 2001; Mirenda et al., 2005; Walker and Abbas, 2002). Alternatively, the expression of inhibitory receptors such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (Perez et al., 1997; Walunas and Bluestone, 1998) or programmed cell death 1 (PD-1) (Lechner et al., 2001) have been implicated in anergic rendering of T cells as a mechanism of tolerance induction. Expression of CTLA-4 appears to be necessary for preventing autoimmunity (Tivol et al., 1995), and it is thought that PD-1
functions by inhibiting cytokine secretion and promoting cell cycle arrest (Freeman et al., 2000; Latchman et al., 2001). Importantly, in respect to anergic rendering as a form of tolerance, although chronic antigen exposure may render T-CD8 cells unable to proliferate or produce cytokine *ex vivo*, the anergic state may be reversible upon transfer into an antigen-free environment, resulting in full recovery of responsiveness *in vitro*. This strongly implicates chronic antigen stimulation as a causative factor responsible for the induction of regulator mechanisms that limit T cell functionality.

Complex intracellular mechanisms, involving defects in several different signaling cascades, drive the observed characteristics of T cell anergy. These mechanisms may include TCR downregulation, decreased expression of ZAP-70, inefficient Ras activation, and defective phosphorylation of downstream signaling molecules (Adams et al., 2004; Dubois et al., 1998; Li et al., 1996; Schwartz, 2003). Additionally, TCR signaling without co-stimulation leads to calcineurin dependent induction of anergy-specific ubiquitin ligase genes, including Itch, Cbl-b, and GRAIL (Bachmaier et al., 2000; Gronski et al., 2004; Jeon et al., 2004). Depending upon which signaling pathway is affected, anergy may manifest in different ways, and it has been observed that activation and expansion of self antigen-specific T-CD8 cells can be uncoupled from effector function. For example, in a mouse model expressing a tumor antigen as a self-antigen, endogenous T-CD8 cells failed to proliferate in response to antigen, but harbored cytolytic activity as well as the ability to make IFNγ. Anergy in these cells was attributed to inhibition of Ca++ flux, ERK1/2 and Jun kinase signaling, indicating that tolerance may differentially affect T cell expansion and acquisition of effector function (Ohlen et al., 2002). In another mouse model of abortive activation/deletion in response to self-antigen in the pancreas (Hernandez et al., 2001), the combination of antigen and B7 co-stimulation (signals 1 and 2) and CD40 triggering promoted proliferation and survival of T-CD8 cells, but inefficient levels of IL-12 (signal 3) contributed to the inability of these cells to produce IFNγ (Hernandez et al., 2002). Also implicated in the maintenance of T-CD8 cell anergy is the negative regulatory molecule CD5, known to regulate TCR signaling (Azzam et al., 2001; Hawiger et al., 2004; Stamou et al., 2003; Tarakhovsky et al., 1995). Collectively, the current data support a model of T cell anergy in which persistent engagement of the TCR promotes continuous downregulation of TCR signaling pathways via activation of various anergy-inducing genes.
IV. **T-CD8 Cells and Tumors**

A. **Tumor Antigens**

The first approach to identifying T-CD8 cell-recognized human tumor antigens was established by Boon and colleagues (van der Bruggen et al., 1991), and made use of antigen-loss tumor cell variants transfected with cDNA isolated from tumor tissue and cytotoxic T-CD8 cell clones. The second biochemical approach was introduced by Ramensee and colleagues, and used acid elution of antigenic peptides bound to tumor cell-derived MHC class I molecules (Falk et al., 1990; Falk et al., 1991b). Ultimately, the identification of lymphocytes capable of recognizing tumor antigens *in vitro* provided the necessary reagent for the molecular identification of cancer antigens, and T cell defined antigens have now been characterized in a large variety of tumor types, in both mice and humans (Boon et al., 1997; Rosenberg, 1999; Van den Eynde and Boon, 1997; Van den Eynde and van der Bruggen, 1997).

Antigens associated with tumors fall into five basic categories, including (i.) cancer testis antigens (MAGE, NY-ESO-1); (ii.) differentiation antigens (tyrosinase, pmel17/gp100); (iii.) overexpressed proteins (MUC-1, CEA, PSA); (iv.) mutated proteins (caspase-8, β-catenin); and (v.) tumor viruses (SV40 T antigen, HPV16 E7). Examples of such tumor-associated antigens (TAAs) have been identified on a variety of malignant melanomas and epithelial tumors, including breast, pancreatic, ovarian, colon, and prostate cancers, and these antigens may be targeted for immune attack. Many TAAs derive from proteins shared between multiple tumor types, and these antigens have been elucidated by virtue of the fact that they elicit an immune response in tumor bearing hosts, but not in healthy volunteers (Stevanovic, 2002). Additionally, some TAAs elicit responses in normal individuals as well as in cancer patients (Romero et al., 2002a; Romero et al., 2002b).

The first three defined antigens that could be recognized by human tumor infiltrating lymphocytes (TILs) were gp100, MART-1, and tyrosinase – all normal non-mutated proteins expressed in both melanomas and in normal melanocytes (Kawakami et al., 1994; Kawakami et al., 1995; Robbins et al., 1994). The discovery of tumor antigen whole protein sequences in combination with the knowledge of HLA allele-specific peptide motifs (Falk et al., 1991b) ultimately facilitated the prediction and identification of T cell tumor epitopes in various tumor
tissue types (Celis et al., 1994; Rotzshke et al., 1991). Armed with the knowledge that tumors bear cell-surface markers towards which T-CD8 cells may be targeted, translational investigations have sought to identify and expand tumor-specific cells in vivo in order to screen for predicted peptide epitopes. These studies confirmed that circulating peripheral blood T cells reactive with tumor epitopes could be isolated from patients that exhibited tumor regressions induced by immunotherapy such as IL-2 administration. Such strategies enabled the rapid identification of probable immunogenic T-CD8 cell epitopes that could serve as targets of immune-mediated tumor regression (Scheibenbogen et al., 2002). Indeed, several defined MHC class I epitopes derived mainly from melanoma antigens are currently in use in clinical trials of active vaccination (Van Der Bruggen et al., 2002; Wang et al., 2002a).

B. Anatomy of the T-CD8 Cell-Mediated Antitumor Response

The anti-tumor response occurs in sequential phases that develop in the context of lymphoid and non-lymphoid tissues. While priming of naïve lymphocytes occurs mainly in secondary lymphoid organs most proximal to the site of tumor growth, effector functions are exerted at the site of the tumor cell bed. The spatial and temporal organization of anti-tumor T-CD8 cell responses has predominately been defined in mice challenged with transplantable tumor cells expressing a defined model tumor-associated antigen and adoptively transferred with T cell receptor transgenic (TCR Tg) T cells specific for the model TAA (Pape et al., 1997a).

The results from studies in mouse tumor models have accentuated the importance of tracing tumor-specific T lymphocytes in various anatomic locations during different phases of tumor growth. Mescher and colleagues found that following intraperitoneal injection of tumor cells, antigen specific T cells were activated in the spleen and LNs, but clonal expansion occurred upon re-localization of T cells to the peritoneal cavity – followed by limited tumor control and T-CD8 cell migration back to secondary lymphoid organs (Kedl and Mescher, 1997; Shrikant and Mescher, 1999). Marzo et al. showed that tumor antigen-specific T-CD8 cells initially proliferated in draining LNs before localizing to the tumor bed (Marzo et al., 1999). The available data depict a model whereby tumor antigens are continuously transported to the most proximal peripheral lymphoid tissue (tumor-draining lymph nodes) by antigen loaded APCs (most likely DCs) and presented to naïve T-CD8 cells. Alternatively, tumor cells themselves may migrate to the DLN. The efficiency of tumor antigen cross-presentation and the maturation state of the APC ultimately determines potential T-CD8 cell activation, proliferation,
differentiation, and migration out of peripheral lymphoid tissues and into the tumor site (Figure 1) (Zimmermann et al., 2005).

C. Antigen Presentation in Tumors

Although some tumor cells are capable of directly priming a T cell response (Ochsenbein et al., 2001; Wolkers et al., 2001), most tumors lack co-stimulatory molecules. Additionally, the presence of tumor cells in lymph nodes usually indicates metastasis and negative prognosis. Thus it is generally agreed that direct presentation by tumor cells is not the predominant pathway by which tumor antigens initiate T-CD8 cell responses (Huang et al., 1994). Rather, tumor antigen recognition is proposed to occur via cross-presentation by bone marrow-derived APCs such as DCs in a TAP dependent manner, and cross-presentation by such cells has been shown for both foreign and self-antigens (Huang et al., 1994; Kurts et al., 2001; Sigal and Rock, 2000). Dendritic cells may take up exogenous antigen from dying cells or may acquire antigen from live cells for cross-presentation (Harshyne et al., 2001; Iyoda et al., 2002; Liu et al., 2002b), and it has been proposed that one explanation for the failure of tumors to elicit antigen specific T-CD8 cell responses in patients is due to the absence of functional DCs at the tumor site (Chaux et al., 1996).

Depending on the presence or absence of immuno-stimulatory conditions during interaction of a naïve T-CD8 cell with an APC, cross-presentation of tumor antigens may lead to either robust activation (cross-priming) or tolerance (cross-tolerance), and the CD8+ DC is responsible for the induction of both pathways (Belz et al., 2002; Heath and Carbone, 2001a, b; Huang et al., 1994; Kurts et al., 1996; Nguyen et al., 2002; Sotomayor et al., 2001). It has been proposed that the delivery of additional signals by the DC is required in order to activate tumor-specific T-CD8 cells, as opposed to induction of anergy or deletion (Albert et al., 2001; Blankenstein and Schuler, 2002; Shortman and Heath, 2001). The anatomical localization of the tumor-antigen presenting DC may also play a role in determining which pathway is chosen. As LN-resident DCs generally harbor a more immature phenotype, under steady-state conditions, these DCs might induce anergy when presenting tumor antigen that drains specifically to a LN (Haanen et al., 2000). Additionally, the half-life of LN-resident DCs is relatively short – with turnover occurring over 3-5 days (Steinman and Cohn, 1974), and it is logical to surmise that unless an inflammatory signal occurs within the LN, DC that permanently reside in secondary lymphoid organs present tumor antigen that drains to LNs in a tolerizing
fashion. Thus, therapeutic interventions designed to stimulate and induce DC maturation offer promise for enhancing tumor antigen presentation that leads to effective tumor-specific T-CD8 cell activation (Figure 7).

D. Effector Mechanisms of T-CD8 Tumor Rejection

The efficacy of T-CD8 cell tumor rejection appears to be dependent on several variables, including the frequency of tumor-specific T cells (Lyman et al., 2004), the affinity of the TCR for target tumor antigen (Overwijk et al., 2003), tumor burden (Cordaro et al., 2000; Garbi et al., 2003), and the level of antigen expressed by the tumor cells (Spiotto et al., 2002). Mechanisms of T-CD8 cell control of tumors overlap with mechanisms of attack against infected cells, and include perforin and Fas/FasL mediated cytolysis of tumor cells, as well as production of IFNγ and TNFα (Blankenstein, 2005; Sivinski et al., 2002). While T-CD8 cells may function to reject tumors by direct lysis, the efficacy of anti-tumor T-CD8 cell responses actually correlates better with cytokine production - particularly IFNγ - than with cytotoxicity (Barth et al., 1991; Becker et al., 2001).

Many different models have identified perforin, Fas ligand, IFNγ, and TNFα as important effector mechanisms required for tumor rejection using mice that are deficient in one or more of the genes for these cytokines (Blankenstein, 2005). In most systems, IFNγ appeared to be indispensable for successful tumor elimination (Girardi et al., 2004; Hollenbaugh et al., 2004; Kowalczyk et al., 2003; Nanni et al., 2004; Qin et al., 2003), although irradiation of the host prior to transfer or adoptive transfer of large numbers of T cells obviated the necessity for IFNγ (Peng et al., 2000; Poehlein et al., 2003; Winter et al., 2001). The data from these studies indicate that some effector mechanisms may be able to compensate for deficiency of others, and that cooperation between several effector mechanisms most likely occurs during tumor attack.

i. Direct Effects on Tumors

A substantial amount of evidence has demonstrated a critical role for endogenously produced IFNγ in promoting host responses to tumors, evidenced in many cases by the reduced capacity of IFNγ−/− or IFNγ receptor−/− mice to control tumor development compared to wild-type mice (Schreiber and Farrar, 1993). In respect to the effects of intra-tumoral IFNγ, cytokine
FIGURE 7

Maturation Status of the APC Determines Activation vs. Tolerance of T-CD8 Cells Against Self/Tumor Antigens

A. Under steady-state conditions, in the absence of inflammation, APCs that have acquired tumor antigen retain an immature phenotype and may fail to induce a T-CD8 response. Additionally, immature APCs may induce tolerance towards the tumor. Ultimately, tumors progress - uncontrolled by the immune system.

B. During inflammation, immunization or active ligation of CD40 on APCs that have taken up tumor antigen, APCs acquire a mature phenotype, characterized by the expression of co-stimulatory molecules. Such APCs harbor greater potential for eliciting T-CD8 mediated anti-tumor destruction.
FIGURE 7

Maturation Status of the APC Determines Activation vs. Tolerance of T-CD8 Cells Against Self/Tumor Antigens

A. Steady-state immature APC

No inflammation or Immunization

immature APC

naïve T-CD8 cell

TOLERANCE

B. Steady-state immature APC

Inflammation, Immunization, or CD40 mAb

Mature activated APC

activated T-CD8 cell

Anti-tumor response

antigen activation marker
activity may target genes that encode TAP1, MHC class I molecules, and proteasomal subunits of tumor cells, leading to increased tumor antigen presentation (Ikeda et al., 2002). Tumors have been shown to depress levels of MHC class I, permitting evasion of immunosurveillance and rendering tumors less immunogenic. Thus, IFNγ works to induce enhanced expression of MHC class I pathway proteins in the cells of the tumor, thereby augmenting immunogenicity and promoting tumor-specific T cell recognition. The tumor suppressive effects of IL-12 have also been demonstrated to be dependent on IFNγ (Brunda, 1994; Nastala et al., 1994). Additionally, due to the induction of multiple IFN-responsive genes, direct anti-tumor mechanisms mediated by IFNγ include promotion of tumor angiostasis (Girardi et al., 2004; Kowalczyk et al., 2003; Qin et al., 2003), direct anti-proliferative and anti-metabolic effects, and promotion of tumor cell apoptosis (Bach et al., 1997; Boehm et al., 1997; Farrar and Schreiber, 1993). TNFα may also contribute to tumor rejection by inducing tumor necrosis or inhibiting angiogenesis, as well as directly targeting the existing endothelial cells of the tumor vasculature (Stoecker et al., 2000; Wu et al., 2004).

ii. **Indirect Effects on the Tumor Microenvironment**

Although IFNγ and other cytokines may act directly on tumors, it has been demonstrated that tumor cells may not need to express receptors for IFNγ or for TNFα in order for tumor rejection to occur, suggesting that these cytokines target other cells embedded within the tumor microenvironment (Blankenstein and Qin, 2003). IFNγ may inhibit endothelial cell recruitment (Ikeda et al., 2002) or counteract the pro-angiogenic activities of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) that contribute to neovascularization. Furthermore, IFNγ is considered to be a macrophage-activating factor that endows macrophages with the capacity to kill tumor targets via reactive oxygen/nitrogen intermediates, up-regulate cytotoxic ligands (TNFα, FasL, and TRAIL), and increase IL-12 secretion (Singh et al., 1992; Yu et al., 2006).
E. Tolerance to Tumors

When a pathogen invades the human body, the immune system generally responds with great force to eliminate the foreign entity. In contrast, because cancer cell associated antigens are often derived from non-mutated self-proteins, tumor antigens that stimulate any immune response induce in most instances, weak reactions. Thus, the immune system is charged with discriminanting between “that which is to be destroyed” and “that which is not to be destroyed.” The latter set usually includes most self and tumor antigens because self is not generally perceived as foreign or dangerous (Anderson and Matzinger, 2000). In this sense, tumor immunity can be regarded as a form of autoimmunity, and various modes of tolerance are set into motion in order to protect “self” in the tumor-bearing host. As a result, T-CD8 cells with the potential to target tumors are highly susceptible to both central and peripheral tolerance, thereby limiting the T cell repertoire that can be harnessed for anti-tumor activity.

Priming of tumor-specific T-CD8 cells may occur strictly by direct presentation or cross-presentation, or it may occur by both mechanisms depending on the form and anatomical/cellular localization of the antigen. However, for tumor antigens situated in isolated anatomical locations, cross-presentation is most likely primarily responsible for initial activation of naïve T-CD8 cells in the tumor-draining node (Norbury and Sigal, 2003). Under steady-state physiological conditions, cross-presentation of tumor antigens feasibly occurs in the absence of APC maturation, as revealed in numerous models by the immature phenotype of self antigen-bearing DC, culminating in T-CD8 cell tolerance as opposed to T cell priming (Steinman et al., 2000). In line with such observations, cross-priming of tumor antigens by DCs has often been deemed inefficient and incapable of eliciting protective anti-tumor immunity (Ochsenbein et al., 2001; Zinkernagel, 2002). As a possible explanation for these observations, it has been demonstrated that in the absence of inflammation, DCs may take up natural tissue autoantigens and present them in the DLN without deleting antigen specific cells and without inducing autoimmunity, further suggesting that such DCs deliver tolerizing signals to anergize self-reactive T cells (Scheinecker et al., 2002). This effect is most likely not a result of inefficient cross-presentation, but due to insufficient DC activation for cross-priming, as agonistic anti-CD40 mAb can overcome this short-coming and promote robust expansion of tumor specific T-CD8 cells (Hernandez et al., 2001; Kurts et al., 1997; Kurts et al., 1999; Liu et al., 2002b; Ohlen et al., 2002; van Mierlo et al., 2002).
The manifestation of tolerance to tumors may take several different forms. Many cancer models have observed that as a result of tumor/self antigen expression, T-CD8 cells specific for the tumor are deleted from the repertoire by either central or peripheral tolerance mechanisms. However, tolerance may be incomplete, in that residual populations of less strongly reactive (subdominant or immunorecessive tumor epitope specific) T cells survive and can be rescued with immunization (Doan et al., 1998; Doan et al., 1999; Doan et al., 2000; Frazer et al., 2001; Herd et al., 2004; Melero et al., 1997a). Despite tolerizing constraints, it has been reported that both low and high avidity T-CD8 cells specific for self and tumor antigens escape deletion, and can be detected in the periphery of both mice and humans (Cordaro et al., 2002; de Visser et al., 2000; Friedman et al., 2004), and it has been observed in tumor antigen-expressing mice that residual tumor specific T-CD8 cells with high avidity for tumor antigen may be targeted with immunization for effective tumor control (Colella et al., 2000). Additionally, although the majority of the residual population of T-CD8 cells specific for tumor self antigens in both patients and animal models may be characterized by low avidity (Molldrem et al., 2003; Theobald et al., 1997), these T-CD8 cells provide an opportunity for immunotherapy of tumors that express high levels of the tumor antigen (Mayordomo et al., 1996; Morgan et al., 1998; Roth et al., 1996).

In experimental models, it has been shown that tumor-specific T-CD8 cells proliferated in tumor draining lymph nodes at all times during tumor progression, indicating that although tumor antigen is continuously available for cross-presentation to T cells in proximal lymph nodes, the resulting T cell population was unable to exert anti-tumor effects (Robinson et al., 1999). Nguyen et al. demonstrated that in tumor-prone transgenic (RIP-SV40 Tag) mice, tumor antigens were cross-presented by bone marrow-derived APCs. However, this resulted in T-CD8 cell activation that was insufficient to promote tumor rejection, suggestive of T cell tolerance. Elicitation of tumor growth reduction depended upon treatment with an agonistic anti-CD40 mAb, capable of enhancing APC activity (Nguyen et al., 2002). As a possible explanation for these observations, it has been demonstrated that in the absence of inflammation, DCs may take up natural tissue autoantigens and present them in the DLN without deleting antigen specific cells and without inducing autoimmunity, further suggesting that such DCs deliver tolerizing signals to anergize self-reactive T cells (Scheinecker et al., 2002). It has subsequently been demonstrated that T-CD8 cells may respond to cross-presented tumor antigen – provided TAP-competent CD11c+ DCs in the DLN were triggered with anti-CD40 (van Mierlo et al., 2004). Alternatively, antigen presentation by immature DCs may induce
differentiation of naïve self-reactive T cells toward a suppressor/regulatory phenotype (Jonuleit et al., 2001; Roncarolo et al., 2001). Impaired trafficking of tolerant T cells has also been observed, whereby anergic tumor-specific T-CD8 cells remained confined to the tumor-draining lymph nodes (Shrikant and Mescher, 1999). Thus, although DCs in TDLNs may initiate T-CD8 priming, mechanisms of tolerance often prevail, precluding optimal T cell-mediated anti-tumor effects.

Levels of tolerance may depend on the tumor type and manner in which immunity against the tumor was induced. Additionally, tolerance may be reversible with optimally designed immunotherapeutic manipulations. It has been demonstrated that residual low avidity T-CD8 cells specific for a tumor self-antigen in the pancreas responded to vaccination, resulting in rejection of tumor challenge without inducing autoimmunity (Morgan et al., 1998). In another model, peptide boosting of memory cells primed by virus vaccination prevented the deletion of cytotoxic IFNγ-producing memory cells specific for the tumor, but these cells could not control tumor progression. However, functional capacity was regained, indicated by the ability of epitope specific cells to reject tumor challenge upon removal of T cells to an antigen-free environment (den Boer et al., 2004). The ability of such T-CD8 cells to exhibit cytolytic activity towards tumor cells and differentiate into responsive memory cells suggests that under the proper conditions, tumor-reactive cells can be rescued from tolerance, and harnessed in the periphery for anti-tumor reactivity.

V. Immunotherapy for the Treatment of Cancer

A. The Challenge: Tumor Progression in Spite of Immunosurveillance

Studies performed in animal models and in humans have demonstrated that tumor-specific cells do exist, and in humans, the presence of TILs can be predictive of improved clinical outcome. These observations support the idea that tumors are indeed antigenic and immunogenic, that the immune system is alert to cell transformation, and that tumor specific T cell responses develop naturally (Dunn et al., 2004). The identification and enumeration of tumor-specific T-CD8 cells at the tumor site and in the blood of cancer patients has provided compelling evidence for the spontaneous development of anti-tumoral responses (Bras-Goncalves et al., 2003; Yee et al., 1999). Similar methods have shown in Phase I clinical trials
that TAA specific T-CD8 cells are induced by peptide-based vaccines in some patients, but that the majority of the T cell responses to tumors were weak and transient, and unable to control tumor progression (Anichini et al., 1999; Lee et al., 1999b; Pittet et al., 2001; Pittet et al., 1999).

Unfortunately, cancers often progress in spite of tumor immunogenicity. If an anti-tumor immune response is elicited in cancer patients, what then prevents this immune response from obliterating the tumor? One explanation is that in patients with cancer, the endogenous immune response is simply too weak (de Visser et al., 2006). Initially put forth by Paul Erlich in 1909 (Erlich, 1960) and later reintroduced in the 1950's by Burnet and Thomas, the “immune surveillance hypothesis” proposed that the immune system constantly surveys for specific antigens expressed by newly-arising tumors, and rapidly eliminates them (Burnet, 1970; Thomas, 1959). This hypothesis suggests that when tumors are observed to progress, mechanisms have been initiated to allow the tumor to escape or resist immune-surveillance, thereby preventing tumor elimination. One such tactic involves inactivation of tumor cell MHC (Marincola et al., 2000; Travers et al., 1982) or MHC-associated machinery (Restifo et al., 1993; Trowsdale et al., 1980), thus allowing the cancer to evade recognition by circulating T cells. This hypothesis was challenged however, when it was found that cancer developed as frequently in immune-deficient hosts as in immune-competent hosts (Groopman, 1987; Pardoll, 2003; Stutman, 1979). Additionally, through the use of marked tumor antigen specific TCR transgenic T cells, it was observed that tumor cells may induce immune tolerance, limiting the T cell response either by transient activation or by rendering the T cells anergic (Speiser et al., 1997; Staveley-O'Carroll et al., 1998), suggesting that the immune system sees tumors more as self than as foreign. Subsequent investigations reported that the answer is not precisely clean-cut, and have proffered that the endogenous immune response hinges upon the location and properties of tumor cells early in development (Pardoll, 2001, 2003).

It has been proposed that the ability of the immune system to control tumor progression largely depends on the anatomical localization of the tumor, especially proximity to local lymph nodes and tumor-cell expression of co-stimulatory molecules (Ochsenbein et al., 2001; Shankaran et al., 2001). It was thus put forth that the ultimate immune response depends on the site and timing of tumor-specific antigen formation. For example, if the tumor does not generate a novel antigen during the pro-inflammatory phase of development, tolerance dominates. In contrast, if the tumor expresses antigen amidst potent pro-inflammatory factors, a robust anti-tumor response will develop. Such tumors will be eliminated until they potentially
develop specific resistance mechanisms (Pardoll, 2001). When considering the concept of tolerance induction by tumors, it is critical to distinguish between initiation of T cell non-responsiveness through mechanisms of anergy or deletion, and resistance to tumor recognition and killing by activated effector T-CD8 cells. In contrast to T cell directed tolerance induction – which implies failure of immune surveillance under steady-state conditions – the presence of resistance mechanisms that hide the tumor from T cell recognition or inhibit T cell function implies that the tumor was selected for or adapted to survive in an environment in which an active anti-tumor response was initially generated (Pardoll, 2003).

When evaluating the relationship between cancer and the immune system, three stages of balance may be observed, coined by Robert Schreiber as “cancer immunoediting”: (i.) elimination – in which the endogenous immune response controls tumor growth unbeknownst to the tumor-bearing individual; (ii.) equilibrium – in which tumor growth is balanced by tumor destruction, thereby holding the tumor in check; and (iii.) escape – in which the immune response is suppressed and tumor growth prevails (Dunn et al., 2002; Dunn et al., 2004). Explanations for the progressive failure of the immune response to control disease progression include multiple mechanisms of T-CD8 cell tolerance as well as active suppression by the tumor cells themselves. As previously stated, many human tumor antigens are self antigens, and unlike viral proteins, are derived from otherwise normal cells whose biologic function has been altered in such a way that they no longer respond to the body’s homeostatic mechanisms for cell growth control. Although TAAs may be qualitatively distinct in that they are overexpressed, such antigens originate from normal cells whose cell cycle regulatory proteins have gone awry. As the immune system is designed to protect “self,” this is now recognized as a major mechanism by which tumors escape immune-mediated targeting. Thus many newer immunotherapeutic strategies focus on prompting the immune system to see tumors as foreign or dangerous, in order to induce full-fledged attack and ultimate eradication of cancer cells (Kiessling et al., 1999; Salih and Nussler, 2001)

**B. Monitoring the In Vivo Anti-Tumor T-CD8 Cell Response**

Clinical observations in cancer patients with spontaneously regressing tumors have historically supported the hypothesis of a pre-existing anti-tumor response. Since the induction and expansion of tumor-antigen specific T-CD8 cell clones often correlated in some patients with a clinical response and tumor regression, many immune-mediated therapeutic approaches
have focused on targeting tumor-specific T cells for the development and augmentation of tumor-targeted treatment strategies. In order to rate the efficacy of a particular mode of immunotherapy, it is critical to monitor the immune response toward the tumor over time, addressing the potential for initially successful responses to resist tolerance and exert persistent anti-tumor control.

i. **Criteria for Effective T-CD8 Cell Targeting of Tumors**

Evaluation of the anti-tumor response requires assessment of both the T cells - are there sufficient numbers; do they migrate to tumor sites; are they functional; and are they tumor reactive; and the tumor cell - is the antigen expressed and are T cell inhibitors present? In order to address these questions, three criteria have been cited as necessary for the immunologic destruction of established tumors, including: (i.) *in vivo* generation of sufficient numbers of T cells with highly avid recognition of tumor antigens; (ii.) trafficking to and infiltration of the tumor stroma by these cells; and (iii.) activation at the tumor site such that T cells manifest appropriate effector mechanisms such as direct lysis or cytokine secretion that leads to tumor destruction (Rosenberg et al., 2004).

a. **Avidity of Tumor-Specific T-CD8 Cells**

T-CD8 cell effector function often parallels the affinity of the TCR for its cognate peptide-MHC class I ligand and the avidity of this engagement, which ideally leads to signaling cascades that properly activate T cell anti-tumor effector genes. Thus, while sufficient numbers of T cells may expand in a tumor-bearing host, these cells may not be tumor-reactive, due to weak avidity for the respective epitope presented in the context of MHC class I (Alexander-Miller et al., 1996; Gervois et al., 1996; Toes et al., 1996).

b. **Migration to the Tumor Site**

In many cases, rapid tumor-infiltration by T-CD8 cells correlates with tumor rejection and favorable prognosis, and it has been demonstrated that tumors that do not respond to immunotherapy resist T cell infiltration (Mihm et al., 1996; Ochsenbein et al., 2001; Speiser and Ohashi, 1998; Underwood, 1974; van Nagell et al., 1978; Watt and House, 1978). As binding to vascular endothelium is a critical step in lymphocyte extravasation, the adhesion of TILs to
endothelial cells in excised patient tumors and peripheral LN has been assessed alongside analysis of homing receptor expression on both TILs and tumor vasculature. All TILs strongly expressed α-integrins, LFA-1, E-selectin, and CD44, whereas only low-level L-selectin expression was detected. Tumor vasculature showed activation based on elevated levels of ICAM-1, E-selectin, VCAM-1 and PNAd (peripheral node addressin) (Springer, 1990). Additionally, TILs demonstrated enhanced binding to tumor vasculature compared with other endothelial specificities, suggesting that TILs possess certain trafficking properties endowing them with the potential to home back to tumor tissue following adoptive transfer (Salmi et al., 1995). Mechanisms of tumor-infiltration by T-CD8 cells may also involve expression of chemotactic factors such as lymphotactin and IP-10 in the tumor bed (Huang and Xiang, 2004).

c. **Retention of T-CD8 Cell Effector Function**

It has often been reported in both animal and human studies that exposure of tumor specific T cells to antigen in a tumor-bearing host ultimately leads to a state of antigen-specific non-responsiveness, whereby T cells persist, but lose effector function (Yee and Greenberg, 2002). Possible mechanisms include alterations in signal transduction, production of immunosuppressive cytokines and inhibition by T regulatory cells (Deeths et al., 1999; Deeths and Mescher, 1999; Mizoguchi et al., 1992; Staveley-O’Carroll et al., 1998). Thus, it is important to assess functionality of tumor-specific T-CD8 cells at various time points post-therapy, as T cell frequency does not necessarily dictate anti-tumor reactivity.

ii. **The State of Tumor Specific T-CD8 Cells in Cancer Patients**

The first studies that demonstrated the quantification and phenotyping of tumor-specific T-CD8 cells in metastatic tumors directly *ex vivo* were performed in melanoma patients (Romero et al., 1998). These and other studies found that tumor antigen specific T cell responses may develop in patients with metastatic melanoma, accounting at times for more than 2% of the total T-CD8 cell population (Lee et al., 1999b). Tumor antigen-specific T cells were isolated and analyzed prior to *in vitro* stimulation and expansion. This allowed for the native functional state of the circulating tumor antigen specific T-CD8 cells in patients to be assessed, and demonstrated that these cells were non-cytolytic. Moreover, these cells failed to produce cytokines even after phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation, indicative of functional T cell anergy. In other studies that focused on the T-CD8 cell response
following vaccination with a specific melanoma antigen, tumor-reactive T cells could sometimes be detected in peripheral blood of some patients. The responding T cell populations were generally monoclonal, and rarely correlated with clinical efficacy (Coulie et al., 2002; Coulie and van der Bruggen, 2003). Taken together, there is increasing evidence that potentially tumor-reactive T cells in the peripheral circulation of cancer patients are functionally inactive, necessitating immunological intervention.

iii. **Tools for Monitoring the T-CD8 Response Ex Vivo**

An important parameter in vaccine development and immunotherapy after having defined the antigen and the route of application, is how to optimally monitor a patient’s immune response following immunization or adoptive cellular transfer. Such analysis is possible via the use of specific techniques designed to assess immune cell frequency, quality, proliferative status, and functionality directly ex vivo with limited in vitro manipulation, and these assays have become the hallmarks of measuring anti-tumor immune responses in both humans and experimental animals (Yee and Greenberg, 2002).

a. **Tetramer Analysis**

The development of MHC/peptide tetramer analysis has facilitated identification of antigen-specific T cells directly from peripheral blood (Altman et al., 1996). This technique involves ex vivo labeling of cells with fluorochrome-tagged complexes of biotinylated MHC class I heavy chains folded around a known peptide epitope, representing the natural target of the T cells in vivo. This assay provides a reproducible method of quantifying epitope-specific T-CD8 cells and tracking their frequency over time, and has a sensitivity of 1:1000. While tetramer analysis does not give information regarding T-CD8 cell function, the avidity of the T cell can be measured by the intensity of fluorescence as detected by flow cytometric analysis, reflective of TCR affinity for cognate target antigen. Additionally, a more precise method of measuring functional avidity is based on the rate of decay of tetramer staining, indicative of the duration/stability of binding between tetramer and the TCR (Dutoit et al., 2002; Yee et al., 1999). Tetramers may also be used to sort out TAA-specific T-CD8 cells from a patient’s peripheral blood, tumor-infiltrated lymph nodes, and accessible metastases using Fluorescence Activated Cell Sorting (FACS). These tumor relevant T cells can then be cloned and transferred back to the patient in order to specifically target disease progression (Dunbar et al., 1999).
b. **Phenotypic Assessment**

Naïve T-CD8 cells can be distinguished from activated cells according to the expression of specific cell-surface markers. These include, but are not limited to up-regulation of CD69, CD25 (IL-2 β-chain receptor), CD122 (IL-2 and IL-15 α-chain receptor), and CD44, and down-regulation of the LN-homing molecule, L-selectin (Lee et al., 1999b). The expression of enzymes such as perforin and granzyme can also be detected indicative of potential T-CD8 cell functionality. Additionally, at later time points following immunotherapy, the presence of memory tumor specific T-CD8 cells may be evaluated, based upon expression of the IL-7 receptor (CD127) and re-acquisition of L-selectin.

c. **In Vivo Proliferation**

The proliferation of tumor specific T cells in the tumor-bearing experimental animal host can be detected *in vivo* in several ways. The introduction of bromodeoxyuridine (BrdU) either orally or by injection identifies endogenous cell division, as this thymidine analog becomes incorporated into the DNA of daughter cells and is detected *ex vivo* by cell permeabilization and intra-nuclear staining with mAb against BrdU. A second method - the Lyons-Parrish technique - detects proliferation of T cells (up to 7 divisions) that have been adoptively transferred into tumor-bearing hosts. This method is based upon the sequential halving of the intracellular fluorescent dye, carboxyfluorescein diacetate succimidyl ester (CFSE), during cell division (Lyons and Parish, 1994). T cells are labeled with CFSE prior to adoptive transfer, thus allowing the number of proliferative cycles undergone *in vivo* to be determined upon T cell isolation and measurement of CFSE intensity via flow cytometric analysis (Appendix A).

d. **Intracellular Cytokine Production**

The capacity for tumor-specific T-CD8 cells to produce cytokines in response to antigen can be measured *ex vivo* by stimulating T cells with tumor antigen *in vitro* with the inclusion of Brefeldin A (BFA), which blocks transport of newly synthesized proteins from the ER to the cell surface. This assay measures the functional status of T-CD8 cells, by inducing the production of cytokines but preventing their secretion, thus trapping the cytokines within the cytoplasm.
These molecules are detected by flow cytometry with a fluorescently-tagged antibody that binds the accumulated cytokine following cell membrane permeabilization (Appendix B).

e. **ELISPOT**

The (Enzyme-linked ImmunoSPOT) assay is often used to monitor function of T cells that have been isolated from the peripheral blood of patients. T cells are stimulated with tumor antigen *in vitro* and plate-bound antibodies are used to recognize production of a particular functional cytokine such as IFNγ on a per cell basis.

f. **In Vivo Cytotoxicity**

The ability for tumor-specific T-CD8 cells to destroy cells expressing their specific tumor antigen can be measured *in vivo* in animal models. Syngeneic target cells are pulsed with tumor antigen peptide and labeled with CFSE prior to intravenous injection. Similar frequencies of control peptide-pulsed targets, labeled with a different concentration of CFSE, are also administered. If functional tumor-specific T-CD8 cells are present in the host, they will recognize the MHC-peptide complex and kill target cells expressing the particular epitope. The CFSE+ population that remains following recognition and destruction by tumor-specific T cells is compared to the frequencies of pulsed targets in a control environment where no killing has occurred. This assay indicates the cytotoxic function of tumor-reactive T-CD8 cells within the environment of the tumor-bearing host (Appendix C).

C. **Immunotherapeutic Modalities to Target Tumors**

Therapeutic induction of an anti-tumor response can be accomplished in several ways. The two general categories of tumor immunotherapy are active vaccination of the tumor-bearing host in order to raise endogenous T-CD8 cells against the tumor and passive adoptive transfer of T-CD8 cells specific for the tumor into the tumor-bearing recipient.

i. **Active Vaccination Against Tumors**

Cancer vaccines aim to stimulate the adaptive arm of the immune system directly *in vivo*. The central tenet of anti-tumor vaccination relies on the ability of the immune system to
destroy tumor cells and retain lasting memory provided tumor associated antigens are efficiently recognized. Mouse models of both transplantable and spontaneous tumors have demonstrated sensitivity to vaccination leading to active induction of anti-tumor responses \textit{in vivo}. However, despite the presence of T cells that recognize the tumor, tumor growth and lethality are often unaffected due to inadequate T cell numbers, avidity, or ability to infiltrate the tumor site (Bocchia et al., 2000; Boon, 1983; Rosenberg and Dudley, 2004; Speiser et al., 1997).

\textbf{a. Designing an Appropriate Vaccine Strategy}

For a tumor antigen to be recognized as immunogenic, the antigen by itself or associated immune complex must surpass a certain level of immunogenicity. Under normal circumstances, the level of endogenous immunity against a particular tumor antigen is determined by stringency of tolerance against the antigen, and is often below a critical threshold necessary for efficient anti-tumor immunity. Thus, if one chooses an antigen for vaccine purposes against which immune tolerance is stringently maintained, even a strong adjuvant will not raise the level of immunity above the critical threshold and the vaccine will fail. Similarly, if one chooses an antigen against which immune tolerance is less stringently maintained, but uses a weak adjuvant, the vaccine will also fail. However, if one uses a strong adjuvant to present an antigen against which endogenous tolerance is relatively non-stringent, it will be easier to elevate the level of immunity against that antigen above the critical threshold, and the vaccine will be successful. Thus, according to this theory, the choice of a cancer vaccine depends on the nature of the combination of antigens and adjuvants used (Renner et al., 2000). Additionally, it is important to determine what mode of MHC class I presentation is critical or sufficient for activation against a particular tumor type, as direct presentation, cross presentation and a combination of both have all been shown to be necessary in different tumor systems (Ochsenbein et al., 2001; Otahal et al., 2005; Plautz et al., 2000; Yu et al., 2003).

Prior to the identification of specific human tumor antigens (Coulie, 1997), most attempts at cancer immunotherapy depended upon immunization with intact cancer cells or subcellular cancer cell fractions – crude approaches that aimed to trigger cellular responses and usually resulted in limited success (Rosenberg, 2001b). In 1992, the first melanoma patient was successfully vaccinated with the MAGE peptide – the first tumor-specific T-CD8 cell epitope ever defined (Coulie et al., 2002; Stevanovic, 2002; van der Bruggen et al., 1991). Since the discovery of specific tumor antigen genes and corresponding epitopes, multiple vaccination
approaches have been attempted, including administration of antigens alone, combined with adjuvants, pulsed onto APCs, or coupled with cytokine augmentation (Rosenberg, 2001a). Current studies continue to examine variations on these themes, including immunization with (i.) immunodominant peptides derived from cancer antigens or modified peptides aimed at altering anchor residues to improve binding of the peptide to the MHC molecule; (ii.) recombinant viruses expressing tumor antigens; (iii.) dendritic cells pulsed with peptide; and (iv.) naked DNA. Although varied degrees of success were achieved in these clinical studies, the most positive findings reported only sporadic anti-tumor effects despite elicitation of high levels of circulating peripheral blood T cells that recognized tumor antigens (Engelhard et al., 2002; Renner et al., 2000). Clearly, peripheral T-CD8 cell frequency alone was unreliable as a read-out for clinical efficacy, strongly suggesting that either mechanisms enabling tumors to escape immune attack are readily set into motion following induction of immunotherapy or that the frequency of peripheral blood mononuclear cells (PBMCs) does not truly reflect the situation at the tumor site.

c. Forms of Active Vaccination

1. Whole Tumor Cell Vaccination

Strategies toward patient vaccination can be partitioned into two general categories: (i.) those based on whole tumor cells and (ii.) those that target defined tumor antigens. Historically, tumor cell based vaccines were the first to be developed, since due to the lack of information about specific tumor antigens, the tumor cell appeared to be the best source of antigens for activating the immune system. This traditional approach involved the use of inactivated whole tumor cells or cell-derived extracts (reviewed (Maher and Davies, 2004). Initial disappointments utilizing such vaccines were explained by the observation that immunizing tumor cells were poorly immunogenic and failed to induce long-lasting anti-tumor immune responses in vivo. Therefore, subsequent studies included a variety of immunologic adjuvants in the vaccination or modified the tumor cells in order to increase tumor immunogenicity. Such strategies, which included ex vivo immunomodulatory cytokine gene transfer and hybrid cell fusion, resulted in limited enhancement in patient response (Wang et al., 2001).
2. **Protein/Peptide Vaccination**

In contrast to cell-based vaccines, the primary goal of peptide or protein-based approaches is to identify tumor-associated immunogenic peptides and stimulate the immune response specifically against these antigens. In order to be immunogenic *in vivo* and successfully induce or amplify a target-specific immune response, peptides or proteins must be presented in the context of MHC molecules by APCs. One vaccination method is achieved by direct injection of the peptides into the skin, which requires APCs to take up, process, and present the antigen to naïve T cells in nearby LNs. Alternatively, DCs can be loaded with antigen *ex vivo* and re-administered to the patient. Generally, DCs are isolated from peripheral blood or generated in culture under cytokine (GM-CSF, IL-4) stimulation, pulsed with tumor antigen and administered via the intradermal, subcutaneous, or intra-lymphatic route (Banchereau et al., 2003; Banchereau and Palucka, 2005; Banchereau et al., 2001b). It has been demonstrated *in vitro* that human DCs derived from monocytes and loaded with patient-derived melanoma cells cross-primed naïve HLA-A*201*+ restricted T-CD8 cells with target-specific cytotoxicity against four shared melanoma antigens (MAGE-3, gp100, tyrosinase, and MART-1), implicating the use of allogeneic tumor cell lines as a route to deliver tumor antigens to DCs for vaccination (Berard et al., 2000).

d. **Efficacy and Challenges Posed by Active Vaccination**

Many tumor vaccines have successfully expanded T-CD8 cell responses to appropriate antigens, as demonstrated by the ability to target cancer cells through active induction of specific immune response to TAAs (Coulie and van der Bruggen, 2003; Knutson et al., 2001). Some promising results have been reported in experimental animal models, particularly in the setting of protection from tumor challenge (Morris et al., 2003). However, despite the safety of peptide immunization, most strategies based on this vaccination approach have demonstrated extreme clinical insufficiency, and much data indicates that such therapies actually induce T-CD8 cell tolerance toward designated tumor epitopes (Finn, 2003a; Morris et al., 2003). While a wide range of results have been reported for infusion of *ex vivo* peptide-loaded DCs, this approach seems to be the only formulation that is feasible and safe in mice and man, in addition to being capable of inducing tumor protective immunity mediated by T-CD8 cells (Cerundolo et al., 2004; Mayordomo et al., 1995; Renner et al., 2001). Variations on the theme of utilizing
DCs as therapeutic agents in anti-cancer vaccination, including targeting increased frequencies of DCs to tumors and stimulating activation in DCs presenting tumor antigen, continuously offer future promise for this method of immunotherapy (Banchereau and Palucka, 2005; Cui et al., 2003; Furumoto et al., 2004; Okano et al., 2005).

The future of active immunotherapy for cancer holds specific challenges. A critical disadvantage of single epitope based vaccine approaches is that HLA-restricted T-CD8 cell responses are elicited toward only one antigen. Such monovalent vaccines may play a role in advanced disease, if the targeted antigen is critical to tumor growth. However, as single-antigen vaccines may not supply long-term immunity necessary to prevent relapse, it is important to develop antigenic formulations that target multiple antigens for clinical usefulness. Thus, polyepitope vaccines that contain multiple conjoined minimal HLA-restricted T-CD8 cell epitopes, derived from a variety of tumor antigens in one recombinant construct offer feasible alternatives to increasing a patient’s spectrum of anti-tumor T cell responses (Mateo et al., 1999; Renner et al., 2001; Sherritt et al., 2000).

It has been proposed that T cells generated by cancer vaccines may not destroy solid tumors due to their inability to infiltrate and become activated following encounter with tumor antigen in vivo, and that vaccination may prove to be more effective in treating lymphoid tumors, which often express co-stimulatory molecules and provide easier access for T-CD8 cells in the circulation (Rosenberg et al., 2004). Experimental data also suggests that vaccination is more likely to be effective on small tumor burden, such as minimal residual disease following conventional treatments, or tumors at early stages of disease – which precludes hope for treatment of patients with bulky tumor burden or advanced-stage metastatic disease, or tumors that are inaccessible by surgery and non-responsive to chemotherapy or radiation (Bocchia et al., 2000). Therefore, it has been put forth that based upon identification of new or mutated genes involved in neoplastic events, the opportunity to prophylactically vaccinate susceptible patients against future cancer development will yield more promising results than those targeted at treatment of established tumors (Bocchia et al., 2000; Finn, 2003b; Rosenberg et al., 2004).

In conclusion, save for scattered reports, active vaccination approaches to cancer treatment have met with narrow margins of success, and T cell-directed vaccination against cancer remains at a paradoxical standstill. Although anti-tumor immunization may induce immune responses, these are generally insufficient for mediating tumor regression (Renner et
Active vaccination faces the challenge of priming and inducing a response \textit{in vivo} from a T-CD8 cell population that is either virtually invisible or has already been tolerated by exposure to endogenous antigen upon induction of therapy. Therefore, the party administering the vaccine maintains little control over the events that follow injection of the immunizing agent into a patient. Inadequate immune responses may be attributed to a quantitative problem (failure to induce high numbers of tumor-specific T cells) or a qualitative problem (characterized by poor priming or tolerance). Many studies have named clonal deletion, exhaustion, and senescence as potential reasons for failed vaccination trials. Thus, it is important to monitor patients systematically in order to define how to activate T cells optimally \textit{in vivo} against pre-defined tumor antigens (Rubio et al., 2003), and it has been proposed that patient-tailored vaccination will broaden the window of success for cancer treatment centered on active immunization (Rammensee et al., 2002).

Finally, a key point illustrated by animal studies is the relative ineffectiveness of vaccination in the setting of heavy tumor burden. Consequently, a more rewarding option might be to test vaccines in patients with minimal residual disease. Focus may need to be placed on the development of new generation vaccines that focus on augmenting responses with complementary additions to current immunization protocols (Berzofsky et al., 2001). A further alternative is to combine vaccination with other therapeutic modalities in addition to developing broadly applicable vaccines that target T-CD8 cells to multiple tumor-associated epitopes (Graff-Dubois et al., 2002; Maher and Davies, 2004; Padua et al., 2003).

\textbf{ii. Passive Adoptive Immunotherapy of Tumors}

Adaptive immunotherapy, which describes the infusion of tumor-reactive T cells into patients, has demonstrated promise for the treatment of advanced metastatic disease (Dudley and Rosenberg, 2003; Ho et al., 2003; Klebanoff et al., 2005b). Specifically, adoptive transfer of activated cells, coupled with stimulation, results in tumor destruction in mice and man (Dudley et al., 2002a; Overwijk et al., 2003). In contrast to vaccination, adoptive cellular transfer (ACT) therapies achieve T cell stimulation by activating and expanding autologous tumor-reactive T cell populations \textit{ex vivo} and deliver large numbers of these amplified cells back to the patient, thereby bypassing tolerance mechanisms that limit the activation and expansion of T-CD8 cells \textit{in vivo}. 

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Compared to vaccination studies, more encouraging investigations have demonstrated the ability of adoptively transferred anti-tumor T-CD8 cells to mediate the rejection of large, vascularized tumors in mice under the appropriate conditions of host immune suppression and antigen stimulation. In some experimental models, adoptive immunotherapy using amplified T-CD8 cells has demonstrated greater success than vaccination approaches targeting the same epitope (Romieu et al., 1998). Most importantly, exquisitely-tailored adoptive cellular transfer protocols have yielded clinical success for patients bearing highly metastatic and aggressive cancers. A chief advantage of an adoptive T cell therapeutic approach toward cancer is the potential ability to achieve better control over the quantity and quality of the T cell response (Dudley and Rosenberg, 2003; Gattinoni et al., 2006).

a. **Challenges Posed by Adoptive T-CD8 Cell Therapy**

Historically, adoptive immunotherapy in humans was plagued by suboptimal response rates, transient response durations, and extreme toxicity associated with the simultaneous administration of IL-2 necessary for maintaining cell survival (Rosenberg et al., 1994b). In animals, successful elimination of experimental tumors similarly required repeated administration of IL-2, adjuvant, and/or immunosuppression of the tumor-bearing host (Granziero et al., 1999; Hu et al., 1993; Overwijk et al., 1998; Rosenstein et al., 1984; Vierboom et al., 1997). It became clear that challenges posed by such therapeutic strategies would need to be identified and overcome including: (i.) optimal *ex vivo* expansion of tumor antigen specific T cells; (ii.) improving the persistence of transferred T cells *in vivo*; and (iii.) augmenting *in vivo* anti-tumor activity (Knutson et al., 2006).

1. **Ex vivo Expansion of Tumor Antigen Specific T-CD8 Cells**

The feasibility of generating T-CD8 cells *ex vivo* is limited by the frequency of antigen-specific T cells in starting bulk cultures, which is low for most tumor antigens. Thus, in order to expand rare populations of tumor-reactive T cells, common practice has been to clone PBMCs, generating T cell clones with a single peptide specificity and affinity. However, this stringent approach greatly limits the effectiveness of adoptive cell therapy, and polyclonal T cell lines have demonstrated superior efficacy in animal models (Cheever and Chen, 1997), and has also shown to be true in cancer patients (Dudley et al., 2002a). It is critical to determine optimal
conditions for ex vivo T cell expansion, as it has also been shown that T cells grown under stimulating conditions that generate phenotypic and functional activation in vitro demonstrate reduced effectiveness in vivo compared to less stimulated cells (Gattinoni et al., 2005b). It is plausible that expanded T-CD8 cells are improperly programmed during in vitro culture due to suboptimal or inappropriate stimulation conditions, such as inadequate or excessive inclusion of cytokines, costimulation or TCR triggering, leading to tumor-specific T cell populations that are deficient in effector function. These cells may be characterized by poor cytolytic activity, inability to sustain in vivo responsiveness to tumor antigen, or elevated susceptibility to deletion (Alexander-Miller et al., 1996; Ho et al., 2002). Thus, the source of antigen-presenting cell, and choice of cytokines and antibodies included in the culture medium is critical for generating the highest quality T cells used for adoptive transfer (Ho et al., 2002; Knutson et al., 2006). Once the T-CD8 cell phenotype functionally relevant for adoptive transfer has been identified, an appropriate strategy for in vitro expansion must be uniquely designed to target the specific tumor of interest. In some cases, freshly isolated tumor-reactive T cells may even prove to harbor superior anti-tumor effectiveness, requiring minimal in vitro manipulation prior to re-infusion (Huang et al., 2005; Robbins et al., 2004; Zhou et al., 2005).

2. **Improving T-CD8 Persistence In Vivo**

Therapeutic efficacy of T-CD8 cell adoptive transfer is very likely to depend upon T cell longevity in the tumor-bearing host. Initial studies that focused on infusion of T cell clones into patients found that the mean survival of T cell clones was 3 weeks or less (Dudley et al., 2001; Dudley et al., 2002b; Yee et al., 2002). A critical T-CD8 cell parameter demonstrated to correlate with patient response is telomere length, whereby infused T cell clones with long telomeres exhibited superior persistence and effectiveness against tumor metastases (Huang et al., 2005). Since infused T-CD8 cells possess a finite lifespan, it is important to generate T cells for transfer under conditions that promote the differentiation of effectors into memory cells that will proliferate and subsequently give rise to new effector cells capable of destroying tumor tissue over time (Wherry et al., 2004). Additionally, infused T cells must be able to resist apoptotic signals potentially delivered by the tumor itself or by other peripheral tolerance mechanisms. It has been found that multiple factors may contribute to T-CD8 cell persistence in vivo, including irradiation, chemotherapy, immunization, and administration of low-dose IL-2 or IL-15 (Knutson et al., 2006). However, as T-CD8 cell persistence does not always correlate with anti-tumor functionality, the identification of optimal methods for achieving long-term
persistence of T cells that also demonstrate potent tumor-reactive efficacy remains an area of intense scientific exploration.

3. **Augmenting In Vivo Anti-Tumor Function**

Ideally, T-CD8 cells transferred into a tumor-bearing host preferentially home to tumors in order to mediate an antitumor response. However, there is very little evidence to support the necessity for specific homing mechanisms to T cells to reach tumor tissues. It is known that culture conditions may influence trafficking, as murine T cells cultured in IL-2 preferentially homed to sites of inflammation, while T cells cultured in IL-15 homed to lymphoid tissues such as lymph node and spleen (Weninger et al., 2001). However, it has also been shown that antigen-specific T cells traffic indiscriminately and ubiquitously throughout the body and antitumor effectiveness is independent of specific homing mechanisms (Palmer et al., 2004). Therefore, it becomes more important to sequester T-CD8 cells at the tumor site. The level of antigen expression on tumor cells may contribute to the containment of T cells in the tumor bed, thereby eliciting continuous activation and anti-tumor responses. Thus, therapeutic protocols must focus on maintaining MHC class I expression levels – an effect shown to highly depend on IFNγ (Knutson et al., 2006; Propper et al., 2003). Other modes of augmenting the anti-tumor reactivity of transferred T cells include immunization; in vivo activation of APCs; elimination of T regulatory cells via irradiation or chemotherapy; and co-administration of cytokines (Knutson et al., 2006). Current investigations continue to define ways in which to enhance adoptive T cell therapy that will broadly pertain to multiple tumor types. However, as different tumors vary in both their intrinsic cellular natures and their anatomical locations, the methods used to optimize adoptive therapy may prove to be tumor specific.

b. **Adoptive T-CD8 Cell Immunotherapy in the Clinic**

In clinical practice, the adoptive immunotherapeutic approach is best illustrated by treatment of hematological malignancies with allogenic stem cell transfer, or donor leukocyte infusion (DLI). In such a setting, there is compelling evidence that T-CD8 cells deliver a “graft vs leukemia effect” that contributes to therapeutic efficacy (Maher and Davies, 2004; Rooney et al., 2001). However, evidence that such cellular therapies can achieve meaningful control of solid tumors is more limited.
Following the initial report that IL-2 administration could achieve responses in a small number of patients with malignant melanoma, Steven Rosenberg and colleagues eventually pioneered the development of adoptive T-CD8 cell immunotherapy protocols for solid tumors (Gattinoni et al., 2006; Rosenberg, 2004). The investigations conducted by the Rosenberg laboratory best describe the methodical approach towards identifying the components necessary for achieving success following administration of an adoptive cellular therapeutic regimen.

Preliminary studies by this group found that although tumor peptide immunization generated high levels of melanoma TAA-specific T-CD8 cells, T cell frequency did not correlate with clinical responses (Rosenberg, 2001b). Thus, the group turned to the adoptive cellular transfer modality of immunotherapy. In order to overcome challenges posed by ineffective clone generation, clinical attention focused on the development of tumor antigen-specific T cell lines from enriched sources such as tumor infiltrating lymphocytes, and techniques were developed to expand these TILs in vitro. The investigators found that TILs are enriched for T-CD8 cells specific for known melanoma antigens, such as MART-1 and gp100. When TILs were infused into patients, a modest improvement in response rate became apparent, but success was hampered by poor in vivo persistence of the transferred cells (Rosenberg et al., 1994b).

After years of attempting to define the optimal protocol based upon autologous TIL infusion into melanoma patients that met with similar limited therapeutic success (Dudley and Rosenberg, 2003; Dudley et al., 2001), Rosenberg and colleagues ultimately reported that complete regression of multiple metastases was achievable in advanced-stage melanoma patients that had failed all traditional treatments (Dudley et al., 2002a). In this protocol, patients received adoptive transfer of TILs that had been expanded ex vivo for a short-term in culture, combined with a non-myeloablative, lymphodepleting chemotherapy regimen (cyclophosphamide and fludarabine) and administration of low-dose IL-2 (720,000 IU/kg 3X/days for 12 doses). It was reported that 50% of the patients experienced complete regression, while the others demonstrated mixed responses – clearly the first successful report of this type of combination therapy for the treatment of solid malignancy in patients. Similar results were observed in future studies, and it was further found that clinical effectiveness highly correlated with persistence and telomere length of tumor-reactive T-CD8 cells (Huang et al., 2005; Robbins et al., 2004; Zhou et al., 2005).
Additional developments have been made in the application of gene transfer-based strategies to target T-CD8 cells to tumors. These include the use of artificial APCs that permit the \textit{in vitro} expansion of tumor-specific T-CD8 cells (Latouche and Sadelain, 2000). Alternative approaches, which involve genetic modification of T-CD8 cells, using chimeric antigen receptors (Eshhar, 1997; Gong et al., 1999; Willemsen et al., 2003) or introduction of novel TCRs with a specificity for a defined tumor-associated peptide-MHC complex, retarget T cell specificity to a chosen tumor antigen, and have also yielded some clinical success (Morgan et al., 2006). While these recent studies demonstrate the potential for tumor-specific T-CD8 cells to target cancer in patients, it is important to recognize that the most successful studies have been infrequent and limited to certain tumor types, namely melanoma, one of the few human tumors that induces detectable endogenous tumor-infiltrating lymphocytes. It thus becomes essential to apply the information gained in these studies to the treatment of other tumor types, in order to identify both common and novel mechanisms applicable to the immunotherapy of multiple forms of cancer.

c. \textbf{Adoptive T-CD8 Cell Tumor Immunotherapy in the Laboratory}

Clinical efforts using biologic therapy are largely based on mouse models, where the prevention of tumor implantation and growth is often the measure of success. The use of these pre-clinical mouse models of tumor disease has allowed for dissection of the different phases of the anti-tumor response and analysis of the kinetics and anatomy of tumor-specific T cell responses (Egorov, 2006). However, prevention models are not generally applicable with respect to the treatment of patients, as individuals rarely present to physicians for treatment before the initial development of disease. Numerous studies have reported immunotherapeutic approaches that induce complete regression of established solid tumors. However, a great number of these studies are performed with transplanted tumors that are given a limited amount of time to grow in the host, and thus do not truly mimic tumor development in humans. Additionally, many of these immunologic regimens have largely been directed against non-self antigens, whereas the majority of human tumor-associated antigens targeted in clinical efforts are non-mutated self-antigens (Rosenberg, 1999). Thus, in order to properly determine the essential components of cancer immunotherapy, it is crucial that laboratory investigative efforts are performed in relevant models of established cancer. In this way, pre-clinical studies carry greater potential for revealing effective and clinically translational treatment strategies.
1. **Sources of T-CD8 Cells for Adoptive Immunotherapy:**

   **T Cell Receptor Transgenic (TCR Tg) Mice**

   The frequency of endogenous tumor-specific T-CD8 cells in tumor-bearing mice is relatively low, making it difficult to assess changes (activation or tolerance) in such T cells following tumor exposure. This necessitates a source of naïve T-CD8 cells that can be adoptively transferred into mice and analyzed for the response to the tumor, and has been made possible through the use of T Cell Receptor Transgenic (TCR-Tg) donor mice. The adoptive transfer of detectable numbers of TCR Tg T cells into normal syngeneic recipients was developed in the laboratory of Mark Jenkins, and this technique allows for the *in vivo* tracing of T cells infused into recipient mice (Pape et al., 1997a). TCR-Tg mice contain the α and β receptors for a single clonal TCR in their germline, and are often crossed onto the background of Recombinase Activating Gene (RAG) knock-out mice, preventing the development of bona fide T cells specific for other antigens. Thus, TCR Tg mice represent sources of pure clonal T-CD8 cell populations that can be genetically engineered to possess the identical avidity for a particular specific antigen. These clonal lymphocytes can be harvested from secondary lymphoid organs of such mice and adoptively transferred into new hosts - such as tumor-bearing mice - at frequencies high enough to allow for flow cytometric detection with monoclonal antibody (mAb) or tetramer, even in the absence of T-CD8 cell expansion.

2. **Transplantable Tumor Mouse Models**

   Cancer is a progressive disease, arising from normal tissue and developing over time in the context of the host immune system. Many mouse models assess T-CD8 cell effectiveness against transplantable tumors that are injected subcutaneously and allowed to grow for a few days to weeks before administering an immunotherapeutic modality. However, these models cannot be directly translated to a clinical scenario, as the tumor-associated antigen being studied is often a foreign antigen. Some models have attempted to more closely mimic the human situation by inserting self-antigens into the transplanted tumors. A recent study published by a group that focuses a great deal of effort on treating melanoma patients with adoptive immunotherapy in the clinic, developed such a mouse model to parallel their human studies (Finkelstein et al., 2004). This murine model involves subcutaneous transplantation of highly aggressive, but poorly immunogenic B16 melanoma tumors that express the human
The homologue of a highly relevant melanoma TAA, gp100 (pmel-17 in mice) into C57BL/6 mice. The investigators found that similar to their reports in clinical trials, three elements could be defined as necessary for induction of regression of large, bulky solid tumors: (i.) adoptive transfer of tumor-specific T-CD8 cells, (ii.) T cell stimulation through antigen-specific vaccination, and (iii.) co-administration of a T cell growth and activation factor such as IL-2 or IL-15 (Klebanoff et al., 2004; Overwijk et al., 2003).

Other groups have assessed the response to tumor challenge with tumor cells that express a protein identical to an antigen expressed by transgenic mice under a tissue specific promoter, i.e. RIP-OVA (Ovalbumin expression by pancreatic islet β cells) or Insulin-HA [(transgenic expression of Influenza Hemaglutinin (Flu-HA)] as a self- Ag on pancreatic islet β cells followed by challenge with renal carcinoma cells transfected with Flu-HA (Renca-HA cells) (Morgan et al., 1998). In these situations, the tumor antigen poses as a self-antigen, allowing for the anti-tumor response to be evaluated in the context of tolerance. Alternatively, investigators have attempted to assess human T cell responses by using humanized HLA-A*0201 transgenic mouse models and challenging mice with tumor lines expressing relevant epitopes, such as HPV16 E7 (Eiben et al., 2002). Despite attempts to modify transplantable tumor models to make them more clinically relevant, such models can never truly recapitulate a real-life situation, as most human cancers develop insidiously from what was once a normal cell, and the host immune response is very likely to be altered due to persistent exposure to tumor antigens.

3. **Spontaneous Tumor Mouse Models**

In cancer patients, tumors arise from a previously normal cell that expresses self-antigens. Thus, the most clinically relevant model useful for mimicking the human situation is a mouse in which tumors arise spontaneously and develop progressively. This has become possible through the development of transgenic mice, in which alteration of the germline DNA, often under a tissue-specific promoter, leads to the development of spontaneous tumors. The causative genetic manipulation may represent the insertion/overexpression of an oncogene such as that which encodes a viral oncoprotein (Hanahan, 1990) or the deletion/mutation of critical regulatory genes such as p53 (Abate-Shen, 2006). Examples of such transgenic mouse models include:
A. Mice expressing SV40 Tag Oncoprotein

Numerous spontaneous tumor mouse models have been created utilizing the Simian virus 40 (SV40) large T antigen (Tag) oncogene driven by various tissue-specific promoters, and these models have greatly facilitated investigations targeted toward understanding tumor immunity in vivo. Some of these models are further described below, and include tumors of the pancreas (RIP-Tag lines) (Hanahan, 1985); bone (Line 501) (Marton et al., 2000); prostate (TRAMP mice) (Gingrich et al., 1996); brain (Line SV11) (Van Dyke et al., 1987); retinal pigment epithelium (RPE mice (Anand et al., 1994); lung (CCSP mice) (Magdaleno et al., 1997); and ovary (Line MISII) (Connolly et al., 2003).

B. HER-2/neu mice

HER-2/neu mice express the non-transforming rat proto-oncogene Her-2/neu driven by the mouse mammary tumor virus (MMTV) promoter, and develop spontaneous focal mammary adenocarcinomas that are highly immunogenic and mimic Her-2/neu overexpressing human breast cancers (Guy et al., 1992). Despite significant tolerance to the transgene, low-level Neu-specific immune responses similar to those observed in patients can be demonstrated prior to vaccination (Reilly et al., 2000). This response can be boosted with vaccination, although not to the extent observed in wild-type mice. Using irradiated tumor cell-based and recombinant vaccinia virus (rVV) based immunization, mice can be protected from tumor challenge and spontaneous tumor growth is delayed (Ercolini et al., 2005; Ercolini et al., 2003). These studies showed that despite tolerance in a spontaneous tumor model, it is possible to induce immunodominant epitope-specific T-CD8 cells for successful elicitation of an antitumor response.

C. CEA mice

The carcinoembryonic antigen (CEA) is a self, tumor-associated antigen, which is expressed by different human adenocarcinomas and also serves as a target for activated T cell specific immunotherapy. Similar to humans, CEA expression in mice transgenic for the human CEA gene (CEA.Tg) occurs predominantly along the gastrointestinal tract. In order to create a model of spontaneous tumors, CEA.Tg mice were crossed with mice bearing a mutation in the Apc gene (MIN mice). CEA.Tg/MIN progeny develop multiple intestinal neoplasms, which overexpress CEA and various methods of immunization have shown to correlate with intestinal tumor-reactivity. Thus CEA.Tg/MIN mice represent another clinically relevant model in which
different CEA-based vaccine strategies may be tested against the spontaneous onset of intestinal tumorigenesis (Clarke et al., 1998; Greiner et al., 2002; Kass et al., 1999; Thompson et al., 1997).

D. MUC-1 mice

Human mucin 1 (MUC-1) is an epithelial mucin glycoprotein that is overexpressed in 90% of all adenocarcinomas including breast, lung, pancreas, prostate, stomach, colon, and ovary. In patients with solid adenocarcinomas, low-level cellular and humoral immune responses to MUC-1 have been observed, which are not sufficient to eradicate the growing tumor. Thus, MUC-1 represents a target for immune intervention. Transgenic mice that express MUC-1 and develop spontaneous mammary tumors are tolerant to the MUC-1 tumor antigen and have been used for studies of tumor control following various immunomodulatory interventions (Mukherjee et al., 2003; Rowse et al., 1998).

Such mouse models of neoplasia in which tumors arise spontaneously in a particular tissue, and develop progressively into advanced-stage tumors represent ideal experimental models for the study of immune responses to cancer. Not only can the response by the endogenous T-CD8 cell population that has been exposed to the growing tumor be assessed, clonotypic tumor-specific TCR-Tg T cells may be adoptively transferred into tumor-bearing mice at various time points during tumor growth, and evaluated for susceptibility to tolerance and reactivity toward tumor progression.

VI. Immune escape

Many models of tumor immunotherapy report the ability to either prevent tumor challenge or to induce regression of established tumors. However, very often at the point when most cancer patients seek medical attention, tumors are already of considerable size and may have already metastasized. Thus, it becomes essential to evaluate the potential for the immune system to target large, well-established tumors that are embedded within a stromal matrix. An important consideration for all forms of immunotherapy is the tumor microenvironment, which is frequently poorly conducive to T-CD8 cell function. The range of immune escape mechanisms set into motion following the initiation of an anti-tumor immune response includes alterations in both the tumor-specific T-CD8 cell as well as in the tumor and the tumor microenvironment.
A. T-CD8 Cell Targeted Immune Escape

Through the use of tumor-specific TCR transgenic T-CD8 cell adoptive transfer into tumor-bearing hosts, much evidence has been generated documenting the ability of tumor cells to resist immune-mediated therapy by inducing tolerance to their antigens at the T cell level. Resistance mechanisms targeted toward tumor-reactive T-CD8 cells include aversion of T cell activation within an immunosuppressive tumor draining lymph node; deletion upon tumor encounter; inadequate T cell function due to anergic rendering; clonal exhaustion from chronic antigen stimulation; inhibitory receptor-mediated inhibition; and active suppression by T regulatory cells.

i. Immune-Suppression in the Tumor-Draining Lymph Node

The tumor-draining lymph node is traditionally considered to be the site of priming for T cells specific for tumors, and tumor vaccination has generally targeted central T cell priming in draining nodes. However, many tumors lack lymph vessels - structural defects that potentially contribute to poor or dysfunctional T-CD8 cell activation in the tumor microenvironment (Zou, 2005). Additionally, the tumor-draining sentinel lymph node on the direct lymphatic drainage pathway, which usually harbors primary metastases, often demonstrates significant suppression in its ability to respond to antigenic stimulation. The immune-suppressive characteristics harbored by TDLNs may include production of suppressor cytokines such as IL-10 and transforming growth factor β (TGFβ) by tolerizing APCs and T regulatory cells. Thus, down-regulation of draining node immunity is likely to affect susceptibility of TDLNs to tumor metastases and thwart the initial activation of naïve tumor-specific T cells (Cochran et al., 2006; Hargadon et al., 2006; Mullins and Engelhard, 2006; Munn and Mellor, 2006; Shu et al., 2006).

ii. Deletion of Tumor-Specific T-CD8 cells

The inability to cure established tumors has been suggested to result from impaired immune responses due to tumor-specific T-CD8 cell death (Finke et al., 1999), and the expression of FasL by tumors remains a potential candidate for eventual T cell apoptosis and induction of deletional tolerance (Hahne et al., 1996). It has been observed that due to MHC class I restricted presentation, adoptively transferred TCR Tg T cells specific for self-antigen are
deleted from the peripheral pool of circulating lymphocytes following an initial period of activation (Kurts et al., 1996; Kurts et al., 1997). The particular mode of tolerance induction has been postulated to depend upon levels of antigen expression - which determined deletion (high-dose antigen) from ignorance (low-dose antigen) (Kurts et al., 1998b; Kurts et al., 1999). Increasing evidence suggests that the effect of persistent antigen on the fate of naïve T-CD8 cells during peripheral tolerance is determined by the strength of interaction between TCR and peptide-MHC complex, whereby high doses of chronic antigen promoted T cell anergy, and low doses led to clonal deletion (Redmond et al., 2005; Rocha et al., 1995). Thus, in early stages of tumor growth, T-CD8 cells may be the most susceptible to deletion, while in later stages, enhanced levels of tumor antigen derived from heavy tumor burden induces T cell anergy.

iii. Inhibitory Receptor-Mediated T-CD8 Cell Anergy

While weak anti-tumor immune responses have sometimes been ascribed to limitations in the activation phase due to inadequate antigen presentation and priming of T cells in tumor-draining lymph nodes (Chen, 1998; Hanson et al., 2000; Levitsky, 2000; Ochsenbein et al., 1999; Speiser et al., 1997), another escape mechanism concerns tolerance induction at the tumor site. It has been shown that the site of successful T-CD8 cell priming can be dissected from the site of effector inhibition, whereby APCs in the TDLN adequately induce activated T cells that proliferated and exhibited function. However, tumor-specific T cell inhibition occurred within the tumor during the effector phase of the response, correlative with down-regulation of TCR signal transduction (Nelson et al., 2001).

Triggering of T cell surface receptors such as CD28, OX40, and 4-1BB costimulates T-CD8 cell growth and prevents activated T cell death (Boise et al., 1995a; Boise et al., 1995b; Watts and DeBenedette, 1999). In contrast, other members of the CD28 family of receptors, such as CTLA-4, PD-1, ICOS, and BTLA play a role in controlling the adaptive phase of the immune response (Khoury and Sayegh, 2004; Riley and June, 2005). For example, engagement of CTLA-4, the T cell expressed inhibitory receptor for B7-1/B7-2 may inhibit T cell growth by blocking cell-cycle progression (Krummel and Allison, 1996; Walunas et al., 1996). Programmed death-1 (PD-1) is also expressed on T cells and binds to the cognate ligands B7-H1 and B7-DC. This interaction plays a critical role mechanistically in down-modulating immune responses and maintaining peripheral tolerance through cell cycle arrest and loss of effector function (Carreno and Collins, 2002; Dong and Chen, 2003; Latchman et al., 2001; Nishimura et
The signals delivered by such co-inhibitory molecules form a network to regulate immune responses, by controlling priming, differentiation, and functional maturation of T cell responses. While co-inhibitory mechanisms normally work to maintain normal homeostasis, these receptors also contribute to preventing immune responses following cancer immunotherapy, thereby exacerbating tumor pathogenesis and disease progression (Chen, 2004; Sharpe and Freeman, 2002).

a. **Programmed Death-1 Receptor (PD-1)**

Programmed death-1 (PD-1) is an inhibitory member of the CD28 family that was originally isolated from apoptotic T cell lines, and has been shown to play a critical role in down-modulating immune responses and maintaining peripheral tolerance (Carreno and Collins, 2002; Dong and Chen, 2003; Nishimura et al., 1999; Okazaki et al., 2002; Sharpe and Freeman, 2002). The PD-1 receptor contains tyrosines in ITIM-like motifs, may be expressed on activated T cells, B cells, and myeloid cells (Sharpe and Freeman, 2002) and inhibits T cell responses through distinct and potentially synergistic mechanisms from those utilized by CTLA-4 (Parry et al., 2005). Due to its immune regulatory role, PD-1 deficient mice develop severe autoimmune disease (Nishimura et al., 1999; Nishimura et al., 2001). Additionally, PD-1 mRNA is highly expressed in CD4+CD25+ T regulatory cells and anergic T cells, indicating several mechanisms by which PD-1 may be involved in regulating tolerance (Gavin et al., 2002; Lechner et al., 2001).

b. **PD-1 Ligands**

PD-1 binds two putative ligands - B7-H1 (PD-L1) and B7-DC (PD-L2). B7-H1 is constitutively expressed on T cells, B cells, macrophages, and DCs and is further up-regulated following activation (Ishida et al., 2002; Yamazaki et al., 2002). The expression of B7-H1 is also detectable on non-lymphoid cells, including cardiac endothelium, pancreatic β cells, glial cells in the inflamed brain, and muscle cells (Iwai et al., 2003; Liang et al., 2003; Salama et al., 2003; Wiendl et al., 2003). Expression of B7-H1 on APCs has become increasingly evident, and it has been shown that B7-H1 is highly expressed on resting immature DCs that inhibit T cell activation and induce peripheral tolerance and anergy, suggesting a regulatory role for such DCs (Probst et al., 2005). B7-DC (PD-L2) is expressed constitutively in the liver, lung, and spleen, and is inducibly expressed on DCs and macrophages (Ishida et al., 2002; Latchman et al., 2001; Tseng et al., 2001; Yamazaki et al., 2002). Expression of both ligands has also been
detected on tumor cells from various origins, including ovary, kidney, and brain (Curiel et al., 2003; Dong et al., 2002; Konishi et al., 2004; Ohigashi et al., 2005; Okazaki and Honjo, 2006; Strome et al., 2003; Thompson et al., 2004; Wintterle et al., 2003).

Ligation of PD-1 on T cells leads to cell cycle arrest in G0/G1 phase, and the interaction between PD-1 and B7-H1 results in decreased numbers of cycling T cells and slower rate of division (Latchman et al., 2001). Co-stimulation with soluble anti-CD28 can overcome PD-1 mediated inhibition via augmentation of IL-2 production. However, even in the presence of co-stimulation, PD-1/ B7-H1 interactions inhibit IL-2 production, suggesting that after prolonged activation inhibition dominates. Exogenous IL-2 has been shown to overcome B7-H1 mediated inhibition, indicating that cells may maintain responsiveness to IL-2 (Carter et al., 2002). While both CD4 and CD8 T cell subsets are susceptible to this inhibitory pathway, CD8+ T cells are more sensitive to modulation by PD-1/ B7-H1, due to their inability to produce significant levels of IL-2 (Carter et al., 2002).

c. **Functions of PD-1 in vivo**

PD-1 mediated interactions have been shown to play multiple roles in vivo. It has been demonstrated that the combined expression of B7-H1 on APCs and on pancreatic islets mediates tissue tolerance and protects against autoimmunity (Keir et al., 2006; Okazaki and Honjo, 2006). In acute lymphocytic choriomeningitis virus (LCMV) infection, PD-1 is transiently up-regulated on virus-specific T cells (by day 6 post-infection) but is rapidly down-regulated following clearance (between days 8-15). However, in chronic LCMV infection characterized by persistent antigen expression, PD-1 remains expressed at very high levels, correlating with virus-specific T cell exhaustion. *In vivo* mAb blockade of PD-1 enhanced T cell responses toward LCMV, and restored T cell proliferation, cytokine secretion, and cytotoxicity towards infected cells (Barber et al., 2006).

During chronic HIV infection, virus-specific T-CD8 cells show a reduced capacity to proliferate and produce effector cytokines. These effects have been shown to correlate with up-regulation of PD-1 on HIV-specific T-CD8 cells isolated from human patients. Blocking PD-1 engagement to its ligand with mAb to B7-H1 enhanced the capacity of HIV-specific T cells to survive and proliferate and increased the production of cytokines and cytotoxic molecules in response to *ex vivo* antigen stimulation (Trautmann et al., 2006). Additionally, as PD-1
expression may also contribute to increased apoptosis, B7-H1 blockade led to alterations in the ability of HIV-specific T cells to survive and expand (Petrovas et al., 2006).

B7-H1 expression is also up-regulated on IFNγ stimulated endothelium, leading to down-regulation of T cell responsiveness. One study demonstrated that IFNγ-activated endothelial cells may inhibit T cell activation via B7-H1 expression since mAb blockade of endothelial B7-H1 enhanced functionality (cytokine production and cytolysis) of T-CD8 cells in response to endothelial cell antigen presentation (Rodig et al., 2003).

d. **PD-1 in Cancer**

B7-H1 and B7-DC expression has been found on various tumor cell types, including freshly isolated human melanoma specimens; carcinomas of the lung, ovary, colon, bladder, breast, cervix, liver, head, and neck; and glioblastoma. Additionally, it was shown that tumor cell expression of B7-H1 suppressed cytolytic activity of T-CD8 cells (Hirano et al., 2005; Iwai et al., 2002). The observation that activated T-CD8 cell (2C TCR transgenic cells and human clones) interaction with tumor-associated (mouse p815 and human melanoma lines) B7-H1 led to programmed cell death, implicated another mechanism by which tumors evade immune destruction (Dong et al., 2002). It has been documented that B7-H1 expression renders tumors resistant to immunotherapy and cancer vaccination in mouse tumor models, suggestive that PD-1 and B7-H1 on tumor cells and T cells respectively, shields tumors from T cell lysis (Hirano et al., 2005). Importantly, multiple models have found that tumor eradication can be accelerated by blocking PD-1/ B7-H1 interactions both in vitro and in vivo (Blank et al., 2004; Curiel et al., 2003; He et al., 2004; Hirano et al., 2005; Iwai et al., 2002; Strome et al., 2003). Recently, a strong correlation between tumor cell B7-H1 expression and negative prognosis was demonstrated for cancer patients (Ohigashi et al., 2005; Thompson et al., 2004). These data strongly implicate PD-1/B7-H1 mediated inhibition as a mechanism utilized by tumor cells in an attempt to evade the immune system through the induction of death or anergy in tumor specific T cells (Okazaki and Honjo, 2006).

iv. **Chronic Antigen Stimulation Results in Clonal T-CD8 Cell Exhaustion**

T cells are highly subject to “clonal exhaustion” during persistent antigen stimulation (Kiessling et al., 2000; Welsh and McNally, 1999). This non-responsive state can be
distinguished from anergic rendering in that T cells initially demonstrate effector function, but are ultimately deleted from the T cell repertoire amidst persistent low-level antigen concentrations (Rocha et al., 1995). Clonally exhausted T-CD8 cells are characterized by down-regulated co-stimulatory receptors, compromised secretion of effector cytokines, and reduction in telomere length with subsequent population doublings (Pawelec et al., 2006). Historically, clonal exhaustion has been observed in the context of chronic antigenic stress, particularly in persistent viral infection such as LCMV (Moskophidis et al., 1993; Wherry et al., 2003a). In humans, the expression of CD57 by HIV-specific T-CD8 cells defines replicative senescence and foretells apoptotic T cell death, unrescuable by cytokines such as IL-2 or IL-15, indicative of tolerance-induction during chronic antigen stimulation (Brenchley et al., 2003).

Similar characteristics have been observed in T–CD8 cells isolated from cancer patients (Pawelec et al., 2006). In theory, a heterogeneous endogenous T-CD8 cell response should be capable of maintaining tumor immunosurveillance. However, chronic antigenic stress due to persistent antigen frequently leads to clonal exhaustion. This first manifests as oligoclonal accumulations of dysfunctional cells followed by clonal deletion, or repertoire shrinkage, without a reduction in the actual number of exhausted cells. Attempts by the immune system to maintain responses against persistent antigen may result in accumulation of dysfunctional cells. This reduces diversity of the T cell repertoire for other antigens, as well as receptor diversity for the persistent antigen driving the exhausted state. The eventual result is immunodeficiency, both for new antigens as well as for responses against the tumor (Pawelec et al., 2006).

v. Regulatory T Cell-Mediated Inhibition

T-CD8 cell reactivity may also be controlled by a subset of CD4+ T cells possessing regulatory function, or T regulatory cells (T-regs) (Asano et al., 1996). T-regs are generally characterized by the expression of CD4, CD25 and the transcription factor forkhead box protein 3 (Foxp3) (Ziegler, 2006; Zou, 2006). It has been proposed that T-regs derive from thymocytes that demonstrate high-affinity interaction with self peptide-MHC on thymic epithelial cells (Jordan et al., 2001). T-reg subsets include naturally occurring CD4+ CD25+ T-regs as well as peripherally induced T-regs that produce the suppressive cytokines IL-10 and TGFβ. Once exported to the periphery, these cells suppress proliferation of other T cells in a manner that involves neither antigen specificity nor APCs, but does require cell-to-cell contact (Sakaguchi, 2004; Thornton and Shevach, 2000). T-regs may be induced to initiate peripheral tolerance and
active immune unresponsiveness following the cross-presentation by DCs of antigen derived from apoptotic cells (Ferguson et al., 2002). Multiple studies have documented the contribution of T-reg accumulation in TDLNs or within the tumor bed, to the suppression of tumor immunity in mice (Wang et al., 2006) as well as in humans, correlating with a striking reduction of patient survival (Curiel et al., 2004; Woo et al., 2001).

B. Tumor and Tumor Microenvironment Targeted Immune Escape

Escape from tumor immunity may also manifest in mechanisms elicited either by the tumor itself or by the tumor microenvironment. Tumors that are under immune attack seem to sense the need to avert destruction, and acquire various mechanisms of both hiding from immune-surveillance and actively suppressing immune responses. Additionally, the stroma in which a tumor is embedded consists of fibroblasts, endothelium, and extracellular matrix proteins, and represents a dynamic microenvironment with the potential to drastically influence the anti-tumor immune response. Therefore, when addressing possible explanations for the failure of tumor immunotherapy, it is important to consider not only changes in tumor-specific T cells, but the state of the tumor and tumor bed as well.

i. Tumor

Adequate expression of tumor antigen is critical for proper activation of tumor specific T cells as well as for recognition of tumor targets. It has been shown that naïve T cells ignored solid tumors when cancer cells expressed lower levels of unique antigen, but responded to and rejected solid tumors when cancer cells expressed the same antigen at higher levels (Spiotto et al., 2002). Tumor cells may retain expression of the targeted antigen, but fail to adequately present immunogenic epitopes, due to changes in the antigen-processing machinery. Defects may include alterations in or loss of expression of MHC class I molecules, β2-microglobulin, and molecules associated with TAP or the immunoproteosome (Ferrone and Marincola, 1995; Marincola et al., 2000; Restifo et al., 1993; Rosenberg, 2001b; Vitale et al., 1998; Wang et al., 1996; Zitvogel et al., 2006).

Tumors may also lose expression of potentially immunogenic antigens through endogenous immunoselection or immuno-editing, thus making them invisible to patrolling T cells (Saleh et al., 2001; Shankaran et al., 2001; Urban et al., 1982). Immunotherapy targeted
against a single antigen may promote outgrowth of antigen-loss variants, further supporting advantages of multivalent therapy (Lee et al., 1998; Panelli et al., 2000). Immune-escape mechanisms related to antigen loss are not evident in all tumor types. In fact, while down-modulation of MHC class I molecules has been observed in some cancers, specifically cervical cancers, this effect is reversible and MHC molecules are rapidly up-regulated in response to IFNγ. Additionally, many tumors express even higher levels of MHC class I and processing machinery molecules compared to normal cells (Pardoll, 2003). Although some investigators have reported the specific loss of tumor antigens in relapsing patients as a mechanism for successful escape from immunotherapy, it is still unclear whether tumor antigen loss is a mechanism employed to elude immunosurveillance in the unmanipulated host, (Jager et al., 1997; Ohnmacht et al., 2001; Ward et al., 1990; Yee et al., 2002).

The production of modulatory cytokines, such as IL-10 and TGF-β by tumor cells has also been postulated to contribute to immunosuppression at the tumor site (Beck et al., 2001; Yang et al., 1995). Additionally, tumor immunoprotection from T-CD8 surveillance may include expression of inhibitory ligand receptors (Hirano et al., 2005; Iwai et al., 2002) or anti-apoptotic proteins such as FLIP (Medema et al., 1999) and survivin (Ambrosini et al., 1997). Furthermore, the up-regulation of STAT-3 by tumor cells has been demonstrated to correlate with suppression of pro-inflammatory danger signals and inhibition of DC differentiation (Gabrilovich et al., 1996; Niu et al., 2002; Wang et al., 2002b).

**ii. Tumor Microenvironment**

Tumor stromal cells utilize multiple suppressive factors that inhibit effective immune responses (Gajewski et al., 2006). In some cases, the stroma may prevent T cell entry to the tumor site. Alternatively, the tumor microenvironment may alter the functional potential of phenotypically activated T cells that have successfully infiltrated the tumor mass (Blohm et al., 2002). The tumor stroma may also contribute to inefficient antigen presentation by sequestering antigen and preventing the efficient release of tumor antigens to TDLNs, thereby thwarting T cell priming (Ochsenbein et al., 1999; Ochsenbein et al., 2001). It has been demonstrated that cancer cells expressing low antigen levels were more capable of inducing an immune response when the cells were in suspension versus surrounded by stroma, as is normally the case in solid tissues (Spiotto et al., 2002).
In late stages of tumor development, despite efficient release of tumor antigens, tolerizing conditions may already abound in the tumor microenvironment, potentially disabling the functions of APCs and effector T cells (Palmowski et al., 2002a; Romero et al., 1998). Many malignancies are associated with aberrant cytokine expression patterns by tumor stromal cells, including reduced production of inflammatory cytokines such as IFNγ and IL-12, and overproduction of immunosuppressive cytokines, including vascular endothelial growth factor (VEGF), IL-10 and TGFβ. This cytokine profile quiets the immune response, reduces inflammation, and inhibits T cell destruction of the tumor (Gorelik and Flavell, 2001; Roy et al., 2000; Zou, 2005). The presence of functionally mature myeloid DCs is rare in human tumors, suggestive that DC recruitment, differentiation, maturation, and survival are defective. Thus, stromally produced suppressive factors may inhibit APC immunogenic properties leading to a state of tolerance at the tumor site. Reversal of the potent immunosuppressive effect of TGFβ has been demonstrated in experiments in which TGFβ signaling in T-CD8 cells was blocked. Such manipulations were shown to promote anti-tumor immunity by selectively expanding tumor-specific T cells that retained effective function (Gorelik and Flavell, 2001; Torre-Amione et al., 1990). It has also been shown that tumor-infiltrating macrophages may induce apoptosis in T-CD8 cells via TNFα and nitric oxide mediated mechanisms, leading to elimination of effector T cells from the tumor bed (Saio et al., 2001).

VII. Enhancing Tumor Immunotherapy

Despite the impediments that limit effective immunotherapy of cancer, knowledge of the molecular events that drive anti-tumor immunity, coupled with an awareness of potential mechanisms utilized by tumors to escape immunity, has assisted the development of an arsenal of techniques aimed at augmenting poor T cell-mediated responses (Waldmann, 2003). Some of these approaches focus on enhancing the early activation phase of the anti-tumor response, while others target the removal of suppressive factors that develop later during tumor growth thereby limiting persistent anti-tumor immunity.

A. Optimizing T-CD8 Cells For Adoptive Transfer

In addition to antigen specificity, a number of lymphocyte characteristics can impact cell tumor reactivity in vivo. The maturation state of T-CD8 cells determines in vivo efficacy and
persistence during an immune response, and it has been demonstrated that acquisition of full effector function in vitro paradoxically limits the in vivo anti-tumor efficacy of transferred T-CD8 cells (Gattinoni et al., 2005b; Geginat et al., 2003; Wherry et al., 2003b). Some of the most critical T cell parameters include expression of lymphoid homing and co-stimulatory molecules; production of IL-2; expression of receptors that bind homeostatic/survival cytokines; telomere length; and resistance to apoptosis (Gattinoni et al., 2005b; Gattinoni et al., 2006; Huang et al., 2005; Kaech et al., 2003; Kagamu et al., 1996; Klebanoff et al., 2005a; Zhou et al., 2005). Prolonged culture times used to generate T cell clones for adoptive immunotherapy have shown to yield T-CD8 cells with characteristics contrary to those that demonstrate optimal in vivo effectiveness (Gattinoni et al., 2005b). Thus, alteration of the cytokine component and duration of in vitro expansion cultures might promote development of early effector and central memory T cells, thereby augmenting their anti-tumor reactivity and prolonging their in vivo persistence (Gattinoni et al., 2006; Speiser and Romero, 2005).

B. Immunization

While vaccination has not yielded significantly promising results against spontaneous tumors when administered in isolation, immunization has demonstrated greater potential for enhancing anti-tumor responses when combined with other immunotherapeutic approaches (Cheever and Chen, 1997; Romieu et al., 1998; Rosenberg, 2004, 2005; Rosenberg et al., 2004). Multiple avenues of vaccination are capable of augmenting anti-tumor responses, and include administration of tumor antigen peptide alone or pulsed onto APCs, altered peptide ligands, cell-based immunization, viral vectors, and DNA vaccines. There is great promise for the combination of vaccination and adoptive cellular transfer modalities, and these regimens may be administered temporally in two ways depending on the ultimate therapeutic goal (Bocchia et al., 2000; Melief et al., 2000b; Rapoport et al., 2005)

i. Vaccination/Adoptive Transfer

This strategy is used in order to activate endogenous populations of tumor-specific T-CD8 cells within the tumor-bearing host. These in vivo expanded cells can then be isolated either from peripheral blood, tumor, or lymphoid tissue (TDLNs), cultured in vitro, and infused back into patients for anti-tumor reactivity (Chang et al., 1997; Rosenberg, 2005).
ii. **Adoptive Transfer/Vaccination**

This strategy is utilized as a means to augment the response of adoptively transferred T-CD8 cells, by providing another source of antigen for T cell activation in addition to the tumor itself. As tumors may deliver either weak or tolerizing signals to tumor-specific T cells, the use of cell-, peptide-, or viral-based immunization harbors the potential to circumvent potential tolerance and optimize the anti-tumor efficacy of adoptively transferred T-CD8 effector cells (Overwijk et al., 2003; Rapoport et al., 2005; Romieu et al., 1998; Ryan and Schell, 2006).

C. **Co-stimulation**

Based on the critical role of co-stimulatory signals expressed by APCs in determining the outcome of T cell-dependent immune responses, much effort has focused on introducing such molecules – particularly B7.1 and B7.2 - into vaccines in attempts to enhance *in vivo* priming and anti-tumor activity (Pardoll, 2002).

D. **Cytokine Administration**

Homeostatic cytokines, including IL-2, IL-15, IL-7 and IL-12, greatly impact the *in vivo* proliferation, survival, and function of infused T-CD8 cells (Kieper et al., 2001; Schluns and Lefrancois, 2003). Administration of IL-2 has been shown to greatly enhance the efficacy of tumor-specific T-CD8 cell responses in mice (Klebanoff et al., 2005a; Overwijk et al., 2003; Schonrich et al., 1994; Schrama et al., 2004). Use of IL-2 in the clinic has revealed the necessity to closely monitor cytokine concentration and route of delivery (systemic vs tumor-site). In dose escalation studies using IL-2 administration, renal cell carcinoma patients treated with high-dose IL-2 (700,000 IU/kg, given 3x/day to a maximum of 14 doses) showed significant clinical responses, as high as 24% at the highest dose. However, severe treatment toxicity limited therapeutic effects, stemming predominantly from capillary leak syndrome. (Fisher et al., 2000; Rosenberg, 1988; Rosenberg et al., 1994a). While low-dose IL-2 has shown to be ineffective on its own, it has yielded results when combined with adoptive immunotherapy, unaccompanied by toxic vascular effects (Dudley et al., 2002a; Dudley et al., 2002b; Rosenberg and Dudley, 2004). The administration of other homeostatic cytokines such as IL-7 and IL-15 to cancer patients has been less intensely studied, and future investigations may demonstrate promise for therapeutic efficacy accompanied by reduced toxic side-effects.
E. In vivo Activation of APCs

Recent developments in cancer vaccine and immunotherapy strategies have shown an appreciation for the nature of the APC as central to the induction of effective immune responsiveness toward tumors. These include attempts to activate endogenous tumor-specific T-CD8 cells by more effective introduction of tumor-derived antigen into activated DCs and transfusion of these mature DCs into tumor-bearing hosts (Banchereau and Palucka, 2005; Dallal and Lotze, 2000; Pardoll, 2002; Schuler and Steinman, 1997; Timmerman and Levy, 1999). Maturation of DCs facilitates efficient activation of naïve T-CD8 cells, by virtue of co-stimulatory, adhesion, and cytokine molecule up-regulation (Banchereau et al., 2000).

1. In vivo CD40 Ligation

Among the DC maturation signals that have been implicated in augmentation of T-CD8 cell responses is ligation of CD40 by its ligand CD40L [(Caux et al., 1994; Cella et al., 1996; Grewal and Flavell, 1998; Koch et al., 1996) and reviewed in (Mackey et al., 1998a; Quezada et al., 2004; Toes et al., 1998a; van Kooten and Banchereau, 2000)]. CD40 ligation triggers production of IL-12 and potently stimulates up-regulation of co-stimulatory molecules. Multiple studies in mice have reported promise for anti-tumor immunity following in vivo ligation of CD40 using agonistic anti-CD40 mAbs or recombinant CD40L, indicating that such CD40-based strategies present an alternative to modalities that rely on ex vivo generation of DCs manipulated to express TAAas (Borges et al., 1999; Diehl et al., 1999; Diehl et al., 2000; Dilloo et al., 1997; French et al., 1999; Mackey et al., 1998b; Nakajima et al., 1998; Nguyen et al., 2002; Staveley-O’Carroll et al., 2003; Todryk et al., 2001; van Mierlo et al., 2004; van Mierlo et al., 2002).

Prompted by the success for in vivo ligation of CD40 to augment anti-tumor immunity in animal models, Phase I clinical trials have addressed the safety and pharmacokinetics of recombinant human CD40L (rhuCD40L) in patients with non-Hodgkin’s Lymphoma (NHL) and found that a significant proportion of patients had stable disease while treatment led to complete remission in others. Importantly, systemic (rhuCD40L) therapy was not associated with toxicity aside from transient elevations in hepatic enzymes, which soon returned to normal levels (Vonderheide et al., 2001). Additional clinical studies utilizing humanized anti-CD40 mAbs have
also reported partial responses of multiple myeloma and NHL patients to weekly doses of 2-4 mg/kg of body weight in the absence of side-effects (Advani, 2005), although one study using a different mAb found that dose-limiting toxicity developed at doses as low as 0.3 mg/kg of body weight (Gladue, 2006; Vonderheide, 2006; Vonderheide et al., 2007). Such findings warrant further investigations for CD40 targeted therapy for cancer patients and several other clinical studies are currently in progress (Geldart, 2005; Tong and Stone, 2003; Vonderheide, 2007).

2. Alternative Methods

Additionally, the administration of GM-CSF (Chambers, 2001; van Elsas et al., 1999), or bacterial products that activate innate toll-like receptors (CpG, LPS) (Heckelsmiller et al., 2002), have shown great promise in the activation of immature DCs presenting tumor antigens, thus converting potential tolerance into a robust anti-tumor T cell response (Melief et al., 2000b). These studies support the postulation that the maturation state of the APC regulates tumor-antigen cross-presentation and determines the fate of tumor-specific T-CD8 cell activation and function (Zimmermann et al., 2005).

F. In vivo Activation of T-CD8 Cells

Agonistic mAbs may also be useful for activating T-CD8 cells against tumors in vivo. The agonistic anti-CD137 (4-1BB) has shown success in the enhancement of anti-tumor immunity, while inhibiting the development of autoimmunity (Sun et al., 2002). CD137 is expressed on activated T cells and engagement by its natural ligand leads to growth, cytokine, and maturation. Administration of anti-CD137 mAb replaces 4-1BB ligand normally expressed on DCs, and has been shown to enhance T cell survival (May et al., 2002; Melero et al., 1997b; Wilcox et al., 2004). In the presence of TCR signaling, agonistic CD137 mAb either alone or with peptide vaccination has also shown to stimulate T cell growth, and restore proliferation and effector function of anergic tumor-specific T-CD8 cells, leading to regression of established murine tumors (Melero et al., 1997a; Wilcox et al., 2002; Wilcox et al., 2004).

G. In vivo Blockade of Inhibitory Receptors

In order to dampen a potentially overly robust T cell response and protect the host from autoimmunity, several mechanisms exist to down-regulate the anti-tumor response and inhibit
new T cell activation. The expression of CTLA-4, a molecule expressed subsequent to T cell activation that possesses a higher affinity for B7 than does CD28, delivers inhibitory signals to T cells that oppose the co-stimulatory signals delivered by CD28, thereby inducing cell cycle arrest and inhibition of cytokine production in activated T cells. It has been shown that blockade of this molecule using an antagonistic blocking monoclonal antibody (α-CTLA-4) delivered at the time of vaccination enhances vaccine potency and augments both murine and human anti-tumor immunity (Chambers et al., 2001; Hodi et al., 2003; Sutmuller et al., 2001; van Elsas et al., 1999; Zou, 2005).

Another potential target for inhibitors of immunologic checkpoints is PD-1, a cell surface molecule induced subsequent to T cell activation, which, like CTLA-4, dampens the T cell response upon binding to one of its receptors – B7-H1 (PDL-1) or B7-DC (PDL-2). Cancer cells as well as DCs and other myeloid cells at the tumor site may express B7-H1 or B7-DC, and blockade of PD-1 signaling upon administration of antagonistic mAbs to either PD-1 or one of its ligands has shown to prevent attenuation of the T cell response, thereby augmenting anti-tumor T-CD8 cell efficacy (Barber et al., 2006; Blank et al., 2004; Curiel et al., 2003; Dong et al., 2002; He et al., 2004; Hirano et al., 2005; Iwai et al., 2002; Latchman et al., 2001; Strome et al., 2003).

H. Depletion of T Regulatory Cells

The depletion of T-regs either before adoptive transfer by cell sorting or in vivo with depleting antibodies has shown to improve functionality and effectiveness of tumor-specific T-CD8 cells (Antony et al., 2005; Powell et al., 2005; Zou, 2006). Not only has antibody-mediated depletion of T-regs led to improved tumor reduction in mice (Onizuka et al., 1999; Shimizu et al., 1999), it has also demonstrated clinical efficacy. The FDA approved ligand fusion toxin Denileukin diftitox (Ontak), consisting of full-length IL-2 fused to the enzymatic domain of diptheria toxin, leads to toxin internalization and inhibition of protein synthesis in CD25+ cells, allowing for the in vivo depletion of T-regs in cancer patients (Foss, 2000).

I. Infusion of CD4+ Helper T Cells

The requirement for CD4+ T cell help in promoting T-CD8 cell persistence has been reported in several models (Lyman et al., 2005; Shedlock and Shen, 2003; Sun and Bevan,
Thus, it may be important to include antigen specific CD4+ T cells in expansion cultures to generate optimally primed T-CD8 cells for adoptive transfer, or to co-infuse CD4+ T cells with tumor-specific T-CD8 cells in order to enhance activation and subvert tolerance in vivo.

J. Lymphodepletion

Depletion of host immune cells prior to adoptive cellular transfer has shown in mice to significantly improve the anti-tumor effect of infused T-CD8 cells (Cheever et al., 1980; Gattinoni et al., 2005a; Maine and Mule, 2002; North, 1982). Similar results have also been recently demonstrated in the clinic (Dudley et al., 2005). Lymphodepletion can be accomplished in several ways – with irradiation and chemotherapy as the most commonly employed methods (Gattinoni et al., 2006). The plausible mechanisms that contribute to increased anti-tumor immunity following lymphodepletion include: (i.) depletion of T regulatory cells (Curiel et al., 2004); (ii.) elimination of endogenous T cells that compete for essential cytokines, such as IL-7 and IL-15 and (iii.) enhancement of APC maturation and function (Zhang et al., 2002). Depending on the intensity of treatment, lymphodepletion may either be transient and non-myeloablative - which salvages host bone-marrow components, or myeloablative – which requires bone marrow rescue with Hematopoietic Stem Cell (HSC) transfer (Dudley et al., 2002a; Gattinoni et al., 2005b; Wrzesinski et al., 2007).

i. Irradiation

Both tumor site-specific and total body ionizing radiation possess immunomodulatory therapeutic properties that hold significant promise for augmentation of tumor immunotherapy (Cameron et al., 1990; Dobbs et al., 1981; Greenberger et al., 1996; Miller et al., 2003; Plautz et al., 1996; Schell and Tevethia, 2001; Wang et al., 2005). Irradiation precipitates the release of inflammatory signals from distressed tissues within the tumor milieu, inducing caspase-mediated apoptosis; cytokine and chemokine release; and up-regulation of MHC, co-stimulatory molecules, adhesion molecules, death receptors, and heat shock proteins in tumor, stromal, and vascular endothelial cells. DCs may be attracted to such a microenvironment, and undergo maturation after internalizing apoptotic and necrotic cellular debris, thereby mediating tumor-specific immunity via presentation of processed antigen to T cells (Hashimoto et al., 1999; Lugade et al., 2005; Miller et al., 2003; Nogami et al., 1994).
A tumor microenvironment permissive for T-CD8 cell infiltration is thought to facilitate effective anti-tumor immunity, and it has been shown that irradiation of tumor-bearing mice rendered solid tumors accessible to infiltration by adoptively transferred T cells (Ganss et al., 1999). Furthermore, following irradiation/adoptive transfer therapy, tumor vasculature reacquired normal structure due to induction of IFNγ inducible protein (IP-10). Such remodeling of endothelium synergistically promoted trafficking of T cells to the tumor site, resulting in tumor cell eradication (Ganss et al., 2002). Additionally, it was recently demonstrated in mice that a myeloablative regimen of irradiation combined with HSC transfer promoted the expansion and functionality of adoptively transferred T cells specific for B16 melanoma tumors (Wrzesinski et al., 2007).

ii. Chemotherapy

Cytotoxic drugs may also be used to modify the tumor microenvironment, rendering it more susceptible to an effective anti-tumor immune response. This can be accomplished via direct cytolysis effect of drugs such as cyclophosphamide, doxorubicin, 5-fluorouracil, gemcitabine, and paclitaxel, which enhance antigen presentation by induction of tumor cell apoptosis. Such drugs may also exhibit mechanisms of immunologic synergy, including DC activation through TLR signaling, re-induced expression of down-regulated MHC molecules, up-regulation of co-stimulatory molecules, destruction of T-reg, and modulation of tumor-associated vasculature allowing for increased T cell trafficking and tumor cell interaction (Emens et al., 2005). Since cytotoxic chemotherapy is widely used to treat many malignancies, the integration of tumor vaccines and/or adoptive immunotherapy with standard chemotherapy protocols presents attractive therapeutic strategies. Such examples are illustrated by the enhanced anti-tumor effect of vaccination in Her-2/neu mice when co-administered with cyclophosphamide (Ercolini et al., 2005) and the clinical success achieved with cyclophosphamide and fludarabine mediated non-myeloablative lymphodepletion combined with autologous TIL adoptive transfer into melanoma patients (Dudley et al., 2005). Careful selection of chemotherapy dose and timing of administration in relation to tumor vaccination maximizes the potential to capitalize on the synergistic effects achieved with collaborative treatment modalities.
K. **Combination Therapy**

Given the potential for individual immunotherapeutic strategies to impart a given level of anti-tumor protection that often wanes over time, the concept of combined immunotherapies for cancer treatment is currently receiving significant attention. Combinatorial protocols place confidence in the ability for multiple approaches to synergistically enhance and prolong responses to cancer by fostering the development of tumor-reactive effector and memory responses. Combinatorial protocols may incorporate different strategies, depending on the tumor type, immune escape mechanisms known to hinder anti-tumor reactivity, and the ultimate objective of immunotherapeutic treatment. Candidate modalities may include two or more of the afore-described methods of tumor immunotherapy enhancement, including tumor specific T-CD8 cell adoptive transfer, irradiation, chemotherapy, vaccination, *in vivo* activation of APCs with TLR agonists or anti-CD40 mAb, cytokine administration, inhibitory factor blockade, depletion of regulatory elements, and HSC transplantation (Banchereau and Palucka, 2005; Cameron et al., 1990; Emens et al., 2005; Ganss et al., 2002; Lake and Robinson, 2005; North, 1982; Pardoll and Allison, 2004; Rapoport et al., 2005; Rosenberg, 1988; van Elsas et al., 1999).

L. **Designing an Optimal Immunotherapeutic Strategy**

Although vaccine-induced tumor-specific T-CD8 cell responses may contribute to tumor rejection, the majority of observed effects have been transient in most cases and provided limited tumor protection in cancer patients (Rosenberg et al., 2004; Yu and Restifo, 2002). While clinical results achieved with adoptive immunotherapy combined with vaccination offer greater promise, these regimens still face multiple challenges (Foster and Rooney, 2006; Gattinoni et al., 2006; Yee et al., 2002). As highlighted by data acquired from animal models, in the presence of large tumor burdens, tumor-specific T-CD8 cell responses develop, but are rapidly outnumbered by the tumor (Yu et al., 2006; Zimmermann et al., 2005). Thus, therapeutic strategies that favor the lymphocyte to tumor ratio may provide a favorable advantage to the immune response. It is important that the vaccination strategy promote not only the development of a robust effector T cell response, but also the development of persistent central and effector tumor-specific memory T cells, capable of mounting strong responses in the event of recurrence or metastasis.
In light of data generated in animal models, spatial and temporal development of tumor-targeted treatment strategies and vaccine protocols in humans demands careful re-evaluation, especially concerning surgical regimens and the site, route, and timing of vaccination. As anti-tumor responses mainly develop in the tumor-draining lymph node, clinical practice of removing the sentinel TDLN has controversial value. While LN dissection harbors prognostic value by correlation of lymphoid-infiltrating T cells with metastasis, surgical resection may obliterate natural immune responses by removing the tissue containing the most potent anti-tumor reactivity. On the other hand, TDLNs have also been shown to harbor immunosuppressive characteristics when compared to LNs further along the lymphatic drainage tree (Munn and Mellor, 2006). Thus, surgical/immunotherapeutic combinatorial regimens must be uniquely tailored to accommodate the tumor type and immunological status of a patient.

The choice of anatomical site of vaccination is also important for maximizing anti-tumor responses. While vaccination close to the tumor bed may in theory facilitate T cell trafficking to the tumor, the TDLN may have been conditioned by the tumor, thus hampering the anti-tumor response. It may be more efficacious to vaccinate at a distal site in order to avoid suppressive interference by the tumor. Based upon studies that implicate anatomic priming site in the imprinting of T cells for tissue-specific homing, the route of vaccination – mucosal, subcutaneous, intravenous - must also be considered (Campbell and Butcher, 2002; Dudda et al., 2005; Dudda et al., 2004).

Another important parameter affecting the T cell response to vaccination and resulting anti-tumor immune response is the timing and frequency of immunization. In response to DC vaccination of cancer patients (4x over 6 weeks), although the number of tumor-specific T cells detected in peripheral blood increased, the effect was short-lived (Banchereau et al., 2001a; Palucka et al., 2003). Such transient blood levels of T cells could reflect migration to the tumor or deletion due to overly excessive vaccination (Masopust et al., 2001). Mouse and human studies of vaccination against infectious agents indicate that for an optimal response, primary immunization should be followed by a booster 4-6 weeks later (Kaech et al., 2002; Zinkernagel, 2003). However, these rules may not apply to treatment of chronic disease such as cancer. A host environment exposed to tumor growth may be compared to chronic viral infection, in which T cells become exhausted from chronic antigen presentation (Wherry et al., 2003a; Zajac et al., 1998), whereby reactivation most likely necessitates carefully-tailored vaccination schedules.
Limitations posed by overly enthusiastic treatment regimens have been demonstrated in the Her-2/neu mouse model, whereby intratumoral immunotherapy with IL-12 and GM-CSF was shown to induce transient tumor regression (Nair et al., 2006). However, repeated immune therapy resulted in loss of tumor-specific T cells, increase in T-reg, and progressive loss of anti-tumor efficacy associated with chronic immune therapy, despite enhanced intratumoral IFNγ production. Such findings have important clinical implications for cancer immunotherapy. The induction of anti-tumor immunity may rapidly be counteracted by regulation, and repeated stimulation can result in a progressive loss of therapeutic efficacy due to increased suppressor activity and eventual immune exhaustion. Thus, standard vaccination protocols may have a limited window of efficacy in the established disease setting, implicating the need for the development of protocols that promote long-term anti-tumor T-CD8 cell persistence and heightened responsiveness to reactivation.

VIII. Immunodominance

The extent to which individual T cell clones specific for a particular antigen respond to antigen stimulation in vivo dictates the immunodominance hierarchy to the immunogenic epitopes derived from the antigen, and examples of such immunodominance have been observed in systems involving viral antigens, minor antigens, and tumor antigens. While numerable peptides encoded by a foreign antigen are potential candidates for presentation to T-CD8 cells, only a few are capable of eliciting a response when displayed in context of MHC. Additionally, among the epitopes that bind with enough affinity to induce a response, an immunogenic hierarchy distinguishes these peptides according to the extent of T cell response they elicit, relative to one another. Generally speaking, immunodominant epitopes elicit the most potent responses, while subdominant epitopes induce weak or barely detectable responses in comparison (Figure 8). The overwhelming nature of T cells specific for immunodominant epitopes to stifle immunogenicity of subdominant epitopes has been termed immunodomination and plays an important role in the design of immunotherapeutic vaccines for infection and cancer (Yewdell and Bennink, 1999).
FIGURE 8

Immunodominance in T-CD8 Cell Responses to Antigen

A. A variety of T-CD8 cells may respond to a given antigen. Each T-CD8 clone responds to a single specific epitope derived from the whole antigen. T-CD8 clones differ in their responsiveness to individual epitope, according to the T-CD8 cell frequency elicited upon immunization with wild type antigen. Immunodominant epitopes induce the most potent responses, while subdominant epitopes elicit weak or barely detectable responses in comparison.

B. In the absence of dominant and subdominant epitopes, T-CD8 cells specific for immunorecessive epitopes are revealed.
FIGURE 8

Immunodominance in T-CD8 Cell Responses to Antigen

A.

Tumor antigen → Tumor antigen epitopes

IMMUNODOMINANT

B.

Tumor antigen → Tumor antigen epitopes

DOMINANT/SUBDOMINANT

IMMUNORECESSIVE
A. Potential Mechanisms Responsible for Immunodominance

Most of the current data on mechanisms responsible for immunodominance have been generated from viral systems, such as influenza virus and LCMV (Yewdell, 2006). Observations made from some of these studies have defined peptide binding affinity for MHC class I molecules and subsequent stability of peptide-MHC complexes as critical for establishing hierarchy in T-CD8 cell responses (Deng et al., 1997; Gallimore et al., 1998c; van der Most et al., 1998). The prevalence of a peptide does not automatically render it dominant, as it has been observed that epitopes present in lowest abundance may actually dominate the T cell response. Rather, the stability of association between the epitope and MHC determined its status as the immunodominant peptide. Similar mechanisms of immunodominance appear to apply to T-CD8 responses against bacterial antigens (Busch and Pamer, 1998), minor antigens (Pion et al., 1999), and tumor antigens (Dutoit et al., 2002). Overall, common factors that appear to contribute to the poor primary immunogenicity of subdominant epitopes in multiple antigen systems include insufficient production or presentation of stable peptide-MHC complexes, low frequency and/or sensitivity of T-CD8 cells specific for subdominant epitopes; and interference by T-CD8 cells that recognize immunodominant epitopes (Hudrisier et al., 2001). Vijh, 1997 #96; van der Burg, 1996 #828; Busch, 1998 #850; Pion, 1999 #2252; Chen, 2000 #840; Yewdell, 1999 #235).

i. Peptide-MHC Complex Presentation by the APC

In general, the more stably a peptide binds to MHC class I molecules, the more efficient its recognition by a T-CD8 cell. The affinity of a peptide for MHC molecules can be influenced by amino and carboxy terminal peptide amino acids, as well as by the anchor residues in the peptide that associate with the MHC binding groove. For peptide generation from full-length protein, it has been observed that flanking sequences on an epitope influence its presentation and ultimate immunogenicity (Chen et al., 2004a; Del Val et al., 1991; Fu et al., 1998; Mo et al., 2000). Thus, peptides that have been cleaved and processed to the proper length and possess optimal residues at appropriate locations may demonstrate higher affinity association with MHC class I molecules, thereby contributing to enhanced T-CD8 cell presentation (Engelhard, 1994; Pamer and Cresswell, 1998; Sette et al., 1994; van der Burg et al., 1996).
It has been proposed that the nature of the APC – efficiency of peptide processing and presentation, expression of co-stimulatory molecules, and requirement for CD4+ T cell help - plays a role determining immunodominance. Additionally, the source of antigen contributes to the presentation of epitopes derived from the antigen. For a virus-infected APC, direct presentation of endogenously synthesized proteins may be sufficient for T-CD8 cell activation. However, for antigens derived from tumor cells, the APC must acquire the antigen exogenously, and cross-present the processed epitope to prime a T cell response (Bevan, 1995; Cho et al., 2003; Kammerer et al., 2002; Li et al., 2001a; Norbury et al., 2004; Rock, 1996; Srivastava, 2002a). Furthermore, optimal processing and presentation machinery within the APC (proteasomal cleavage, TAP-mediated transport, trimming by ER peptidases, and association with β2-M) is ultimately responsible for loading of the final antigen-derived epitope onto MHC class I molecules (Serwold and Shastri, 1999). Cytokines, such as IFNγ and TNFα may also contribute to antigen presentation by increasing synthesis of TAP, MHC class I molecules, and immunoproteasomes (Basler et al., 2004; Chen et al., 2001; Momburg and Hammerling, 1998; Pamer and Cresswell, 1998; Tanaka et al., 1997). Surprisingly, although efficiency of antigen processing contributes to generation of competent immune responses, it cannot completely account for establishing immunodominance. A threshold exists, whereby increasing antigen processing efficiency can no longer enhance the magnitude of T cell response (Vijh et al., 1998). Finally, peptide antagonism, in which a more dominant peptide-MHC complex competes for MHC or blocks the agonistic effect of another peptide, may also skew the observed immunodominance hierarchy (Sette et al., 1996; Sloan-Lancaster and Allen, 1996).

ii. Immunodominance at the T-CD8 Cell Level

a. T-CD8 Cell Repertoire

Although abundance of peptide-MHC class I complexes on the surface of the APC may contribute to the observed dominance of an antigenic peptide, it does not explain why some immunodominant epitopes are actually expressed at lower levels than subdominant epitopes (Bousso et al., 2000; Busch and Pamer, 1998). The composition of the T cell repertoire with the potential to recognize a given antigen also plays a role in organization of the hierarchy. T cell characteristics that may contribute to the establishment of immunodominance include the frequency of naïve T-CD8 cells specific for an epitope, the role of tolerance on the elimination of strongly self-reactive T cells, the unique arrangement of the Vα and Vβ chains in T cell clones,
and the intrinsic proliferative or functional capacity of the T cell in response to activation, irrespective of T cell help or co-stimulation (Chen et al., 2002; Yewdell and Bennink, 1999). Triggering of T-CD8 cell activation requires that the TCR bind with sufficient affinity to the peptide-MHC complex presented by the APC. Thus, differences in TCR ligation to peptide-MHC, as well as the number of complexes necessary for efficient activation of a particular T cell clone contributes to the rank of the epitope within the dominance hierarchy of a given antigen (Kageyama et al., 1995; Kedl et al., 2003; Sykulev et al., 1995).

b. T-CD8 Cell Immunodomination

A significant factor controlling immunodominance is the suppression of subdominant epitope specific responses by T cells specific for dominant epitopes, or immunodomination, which has most extensively been studied in mouse models. This concept can best be appreciated by the observation that the removal or mutation of the dominant epitope, its restriction element, or T cells specific for the determinant enhances responses to subdominant epitopes (Doherty et al., 1978; Fu et al., 1998; Pion et al., 1997; van der Most et al., 1997; Van Waes et al., 1996; Zinkernagel et al., 1978). While it has been proposed that immunodomination may relate to prohibition by the dominant peptide on the generation of the subdominant peptide, T cells specific for immunodominant epitopes appear to contribute most significantly to this effect by the suppression of T cell responses to subdominant epitopes (Chen et al., 2000; Pion et al., 1999; Wolpert et al., 1998).

Once an epitope has been properly processed and presented, T cells may compete for access to an APC expressing both dominant and subdominant epitopes, whereby the epitope that successfully engages TCR ranks utmost in the hierarchy (Gruftman et al., 1999; Kedl et al., 2000; Probst et al., 2002). For example, it has been shown that competition between T-CD8 cells limited both primary and boosted responses to subdominant epitopes when vaccination was performed with polypeptide constructs – an effect that was alleviated when single epitopes were isolated into separate viral or cellular vectors (Otahal et al., 2005; Palmowski et al., 2002b). Other potential mechanisms responsible for immunodomination include antigen saturation by the rapid responses of T cells specific for dominant epitopes, which drains antigen load and leaves suboptimal levels for activation of T cells specific for subdominant epitopes (Chen et al., 2000; Nelson et al., 2000; Pion et al., 1999), and systemic suppression of
responses to subdominant epitopes by T cells specific for dominant epitopes (Chen et al., 2000; Yewdell and Bennink, 1999).

B. Immunorecessive Epitopes

While T-CD8 cell responses directed against a given antigen are characterized as immunodominant or subdominant according to the relative frequencies of T-CD8 cells elicited following immunization with wild-type antigen (Yewdell and Bennink, 1999), additional T cell populations specific for immunorecessive epitopes may be induced following immunization with antigens lacking each of the immunodominant and subdominant epitopes (Figure 8) (Tanaka et al., 1989; Urban et al., 1984). The critical distinction to be appreciated between subdominant and immunorecessive epitopes is that T cell responses against immunorecessive epitopes are revealed only in the absence of both dominant and subdominant epitopes. Immunorecessive epitopes demonstrate even less susceptibility to tolerance than subdominant epitopes, and may represent the only residual specific T-CD8 populations in self/tumor antigen-expressing hosts, making immunorecessive epitopes attractive for the targeting of tumor immunotherapy (Lally et al., 2001; Lotz et al., 2004; Schell, 2004; Theobald et al., 1997).

C. Plasticity in Immunodominance

It has been demonstrated that the immunodominance hierarchy for a memory response may not mirror that established during the primary response, and subdominant epitopes that are ordinarily shielded by overwhelming immunodominant responses represent potential targets for protection against subsequent antigen challenge (Belz et al., 2000; Bennink and Doherty, 1981; Bousso et al., 2000; Chen et al., 2004b; Cole et al., 1997; Crowe et al., 2003; Gallimore et al., 1998a; Gallimore et al., 1998c; Gallimore et al., 1998d; Jamieson and Ahmed, 1989; Sandberg et al., 1998; Slifka et al., 2003; van der Most et al., 1996; Van Waes et al., 1996; Wettstein, 1986). This phenomenon may be attributed to the elevation in average affinity for peptide-MHC by TCR following re-challenge leading to preferential outgrowth of a pool of T cells specific for a subdominant epitope in a secondary response (Busch and Pamer, 1999; Savage et al., 1999). It has also been observed in vivo that high-affinity T cell clones specific for a single antigen induced loss of peptide-MHC from the surface of the APC, thereby inhibiting the response of low-affinity clones (Kedl et al., 2002). Additional plasticity of the immunodominance hierarchy has been demonstrated following loss of dominant epitope presentation by MHC class I.
molecules or during chronic antigen stimulation, allowing for T cell responses to subdominant epitopes to be revealed. Such findings highly suggest the potential for alternative vaccination approaches targeted toward subdominant or immunorecessive epitopes to yield effective T-CD8 responses (van der Most et al., 2003).

**D. Immunodominance Related to Self/Tumor Antigens**

Immunodominance in tumor systems may differ from the rules that govern hierarchical dominance in responses generated against foreign pathogens such as viruses, since the T cell repertoire may already have been edited by self-tolerance. Thus, many of the T cells specific for immunodominant epitopes are centrally or peripherally deleted. In contrast, despite the ability for a subdominant or immunorecessive peptide to bind MHC with reasonable affinity, it is possible for thymic negative selection and peripheral elimination to be avoided. This may result from (i.) inefficient antigen processing/presentation or rapid turnover of subdominant peptides derived from self-antigen acquired by APCs compared to sufficient availability of dominant epitopes (Ludewig et al., 2001; Otahal et al., 2005); (ii.) amino acid residues flanking subdominant epitopes that hinder access to the MHC complex (Grewal et al., 1995; Moudgil et al., 1996); and (iii.) competition either among epitopes competing for the same MHC or among different MHC molecules competing for the first available epitope that unfolds (Deng et al., 1997; Sercarz et al., 1993; Sette et al., 1996; Sloan-Lancaster and Allen, 1996), although the last phenomenon is more applicable for MHC class II complexes. Thus, the T-CD8 cell repertoire directed against subdominant self is protected from negative selection both at the time of repertoire acquisition in the thymus, as well as in the periphery, making such cells attractive targets for immunotherapy (Schoenberger and Sercarz, 1996; Slifka et al., 2003).

**E. The Residual Tumor Reactive T Cell Repertoire:**

**T-CD8 Cells Specific for Subdominant and Immunorecessive Epitopes**

At the level of tumor antigen-specific immunity, a central question concerns the nature of the T-CD8 cell repertoire capable of activation. It has been proposed that tumors induce active tolerance among T cells specific for immunodominant antigens, thereby preserving a residual repertoire of T cells specific for antigens that are not as efficiently processed and low-affinity T cell populations exhibiting tolerance toward the tumor (Friedman et al., 2004; Gervois et al.,
Indeed, the expression of tumor antigens as a self-antigen has been shown to alter the immunodominance hierarchy. This phenomenon is exemplified by the appearance of T-CD8 cells specific for new, more dominant epitopes in self-antigen knockout hosts, indicating that T-CD8 cells against the most immunodominant epitopes are eliminated in the presence of self-antigen (Friedman et al., 2004; Lotz et al., 2004; Theobald et al., 1997).

Clinically, it is interesting to note that high-affinity T-CD8 cells specific for self/tumor antigens such as tyrosinase, MART-1, and gp100 are detectable in normal volunteers, indicating that these T cells escape thymic deletion and are not restricted to the repertoire of melanoma patients (Houghton, 1994; Visseren et al., 1995). Additionally, compared to immunodominant viral epitopes with high affinity for MHC, melanoma tumor antigen epitopes exhibit intermediate binding affinity to the MHC groove (Celis et al., 1995; van Elsas et al., 1996). Thus, T-CD8 populations specific for self/tumor antigens that persist in cancer patients most likely represent a repertoire that has already been purged of the most immunodominant tumor epitope-reactive T cells. These findings suggest that T-CD8 cells specific for subdominant or immunorecessive self/tumor antigens are less susceptible to tolerogenic mechanisms, thereby permitting the persistence of both low and high avidity epitope-specific T cells despite the continuous presence of antigen. Such residual tumor-specific T cell populations signify enticing targets for immunization aimed at expanding effector T-CD8 cells that harbor anti-tumor reactivity.

**F. Targeting Subdominant and Immunorecessive Tumor Epitopes for Immunotherapy**

Given the proper activation conditions, subdominant and immunorecessive epitope specific T-CD8 cells represent candidates for successful anti-tumor therapy. Promising results have been demonstrated by attempts to harness tumor-specific T-CD8 cells reactive for such epitopes in tumor-bearing hosts, and indicate that repertoires of at least moderate to high affinity T cells exist and can be activated and expanded both *in vitro* and *in vivo* (Bullock et al., 2001; Colella et al., 2000; Cordaro et al., 2002; Gross et al., 2004; Lally et al., 2001; Lotz et al., 2004; Morgan et al., 1998; Ohlen et al., 2001; Schell, 2004; Touloukian et al., 2003).
Dendritic cell immunization has been shown to break immunodominance in T-CD8 cell responses against minor histocompatibility antigens and synthetic peptide antigens, unveiling reactivity toward subdominant epitopes (Grufman et al., 1999; Sandberg et al., 1998; Wettstein, 1986; Wolpert et al., 1998). Similarly, in respect to cancer, immunization of melanoma patients with DCs pulsed with gp100 tumor antigen peptides revealed functional T-CD8 cells specific for subdominant tumor epitopes that were not visible in the natural repertoire (Tsai et al., 1997). These findings suggest that comparable mechanisms function to activate subdominant or immunorecessive tumor epitope specific T cells following DC immunization of cancer patients.

The role of co-stimulation in the priming of subdominant epitope-specific responses has also been demonstrated. In an EL4 lymphoma model, immunization with tumor cells expressing B7 induced responses to initially silent subdominant epitopes compared to a single immunodominant epitope-specific response induced in the absence of co-stimulation (Johnston et al., 1996). Importantly, the presence of T-CD8 cells specific for the subdominant epitope was necessary for successful tumor elimination.

It has been observed that whole tumor antigens that stimulate potent immune responses can hinder the response against weaker antigens, if the two antigens are situated in close proximity to one another. Such co-vaccination permitted outgrowth of tumor variants expressing only the weaker antigen. However, immunization with individual antigens at separate sites promoted responses towards the weaker antigen, indicating that when strategically executed, immunotherapeutic approaches are capable of subverting immunodominance and eliciting tumor protection (Van Waes et al., 1996). Similar strategies may be applied to epitopes derived from the same antigen, whereby epitope-targeted immunization reveals immune responses against subdominant or immunorecessive tumor epitopes that are undetectable in the presence of whole tumor antigen containing immunodominant epitopes. A few studies have reported that immunization against subdominant tumor epitopes resulted in protection from transplantable tumor challenge in the absence of self antigen expression (Duraiswamy et al., 2004; Feltkamp et al., 1995; Newmaster et al., 1998), or revealed that T-CD8 cells specific for an immunorecessive epitope can be recruited in the context of self antigen expression (Degl'Innocenti et al., 2005; Grossmann et al., 2001; Schell, 2004; Schell et al., 2000). However, the ability of immunorecessive epitope reactive T-CD8 cells to control the progression of spontaneous tumors has only recently been addressed (Ryan and Schell, 2006; Singh and Paterson, 2007).
IX. Simian Virus 40 (SV40)

Tumor viruses provide valuable information regarding the cellular and molecular events that contribute to tumorigenesis. The study of DNA tumor viruses such as Simian virus 40 (SV40) has contributed significantly to our understanding of how viral oncoproteins disrupt normal cell cycle pathways to induce transformation, and has been an attractive tool in medical research due to its high oncogenicity and transforming capacity.

A. General Virology

SV40 is a member of the Polyomaviridae virus family, distinguished by a circular 5.2 kb double-stranded DNA genome composed of 5243 base pairs, and non-enveloped icosahedral virions. SV40 is related to BK virus (BKV) and JC virus (JCV), human polyomaviruses, which bear clinical relevance in immunocompromised individuals. Although healthy people establish immunity to these viruses early in life, BKV and JCV can induce disease under certain conditions, including JCV-induced neurodegenerative progressive multifocal leukoencephalopathy (PML) in AIDS patients, and BKV-induced tubulointerstitial nephritis in kidney transplant patients. Additionally, both JCV and BKV have possible roles in carcinogenesis (reviewed in (Arrington, 2001; Demeter, 1995)). By contrast, SV40 is of monkey origin, but has been identified in specific human tumour cells (Bergsagel et al., 1992; Carbone et al., 1994; Lednicky et al., 1995; Martini et al., 1996). SV40 entered the human population as a contaminant of poliovirus vaccine in several countries between 1955 and 1963, as these vaccines were prepared in primary cultures of rhesus monkey kidney cells (Demeter, 1995; Gazdar et al., 2002).

The SV40 genome is divided into three elements - early coding, late coding, and regulatory regions – and encodes three structural proteins (VP1, VP2, and VP3) and four nonstructural proteins – the 94,000-molecular weight (94K) large T antigen (Tag), the 18K small T antigen (tag), the 17K T antigen, and the DNA-maturation protein - agnoprotein (Figure 9) (Fanning and Knippers, 1992; Zerrahn et al., 1993). During infection, cellular transcription allows for expression of the early coding region as well as Tag, tag and 17K T. Tag is required for expression of the late coding region (VP1, VP2, VP3), as well as for activating transcription form the viral late promoter and participating in replication of viral DNA and virion assembly.
The genome of the SV40 Polyomavirus is depicted, distinguished by circular 5.2 kb double-stranded DNA composed of 5243 base pairs. SV40 genomic DNA is divided into three elements - early coding, late coding, and regulatory regions – and encodes three structural proteins (VP1, VP2, and VP3) and four nonstructural proteins – the 94,000-molecular weight (94K) large T antigen (Tag), the 18K small T antigen (tag), the 17K T antigen, and the DNA-maturation protein – agnoprotein. The early unit encodes Tag, tag, and 17KT. The late unit encodes for VP1, VP2, VP3, agnoprotein (agno) and a pre-microRNA (miRNA). The regulatory region (ori) contains sequences for the early and late promoter and the origin of replication.
FIGURE 9

The Simian Virus 40 (SV40) Genome
The viral capsid is composed of pentamers of VP1. Two minor structural proteins, VP2 and VP3, produced by alternative splicing, bridge the VP1 capsid to the SV40 genome. VP3 is essential for formation of infection particles, and may be involved in virus-cell interactions during post-packaging steps (reviewed in (Ahuja et al., 2005; Tooze, 1981).

The natural host for SV40 is the *Rhesus macaque*, in which virus infection is asymptomatic. It is believed that SV40 infects the terminally differentiated epithelial cells of the kidney. In order to overcome limitations imposed by the absence of cellular proteins required for replication in these growth-arrested cells, Tag and tag cooperate to drive infected cells into cell cycle, allowing for production of progeny virions, and subsequently alerting the host immune defense system. During infection of permissive cells, SV40 virions penetrate the cell membrane and are transported to the nucleus, where early transcription is initiated from the released viral chromatin. Expression of large and small T antigen proteins drive the cell into S-phase, DNA replication occurs, and transcription from the late promoter is initiated. Upon the assembly of mature virions, death of cultured cells is observed 3 days post infection (reviewed in (Ahuja et al., 2005; Manfredi and Prives, 1994; Tooze, 1981).

B. **SV40 Induced Oncogenesis**

Viral replication, virion release and cell lysis do not occur following SV40 infection of rodent cells (Tooze, 1981). Instead, the virus is capable of precipitating neoplastic transformation in cultured cells, as well as inducing multiple types of tumors in rodents, including osteosarcoma, mesothelioma, lymphoma, and choroid plexus neoplasia [(Livingston and Bradley, 1987; Tevethia, 1980) and reviewed in (Arrington, 2001; Rigby, 2001)]. Following infection of primary rodent cells (such as mouse embryo fibroblasts – MEFs), SV40 enters the nucleus where large and small T antigens are produced. However, DNA replication and transcription from the late promoter do not occur. Rather, Tag induces the cells to enter S-phase, ultimately leading to cellular transformation, characterized by acquisition of augmented proliferative and survival potential. If the viral DNA integrates into the host genome such that the early coding sequences are expressed, a stably transformed cell line is established. The characteristics of such transformed cells include immortalization, prolonged capacity to grow in low serum, failure to arrest cell division upon reaching high saturation densities, foci formation in culture, anchorage independence, and ability to from tumors upon injection into rodents [reviewed in (Ahuja et al., 2005; Manfredi and Prives, 1994)].
C. SV40 Large T antigen (Tag)

Expression of large Tag has been determined via genetic analysis to be necessary and often sufficient for transformation by SV40, although small tag may additionally be required. Three main functions that contribute to cellular transformation have been ascribed to large Tag, and include the binding of tumor suppressor pRb, tumor suppressor p53, and the heat shock chaperone hsc70. Additionally, small tag is responsible for binding of the cellular phosphatase, pp2A (Ali et al., 2004; Manfredi and Prives, 1994; Saenz-Robles et al., 2001; Sullivan and Pipas, 2002).

1. pRb

The chief effect of Tag on the Rb family of proteins is through regulation of the activity of E2F transcription factors (Attwooll et al., 2004; DeCaprio et al., 1988; Ewen et al., 1989). Inactivation of Rb-proteins is normally accomplished via phosphorylation to allow for cell cycle progression, leading to transcription of E2F-regulated genes that encode proteins necessary for DNA replication, nucleotide metabolism, and DNA repair. Conversely, de-phosphorylation of Rb leads to inactivation of E2F and cell cycle arrest. During SV40 transformation, the ultimate effect of Tag sequestering of Rb-proteins is to block their ability to regulate E2F, resulting in uncontrolled cell cycle progression (Christensen and Imperiale, 1995; Zalvide and DeCaprio, 1995).

2. p53

The p53 tumor suppressor was identified as a cellular protein bound to large Tag in SV40 transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Normal, steady-state levels of p53 are low, as it is usually bound to the mdm2 gene, where it functions to activate transcription. When levels of Mdm2 rise, Mdm2 binds p53, leading to its ubiquitination and degradation [reviewed in (Bond et al., 2005)]. However, under cellular stress (DNA damage, anoxia) the p53-mdm2 interaction is disrupted, leading to a rise in p53 levels and transcriptional activation of genes involved in apoptosis and cell cycle arrest. During SV40 transformation, Tag engages the DNA-binding motif of p53 and inhibits its ability to bind to regulate gene expression (Bargonetti et al., 1992; Jiang et al., 1993). Tag interaction with p53 stabilizes the protein in an inactive form, ultimately inhibiting its function as a tumor suppressor, allowing for uncontrolled cell cycle progression (Oren et al., 1981). Ultimately, by blocking pRB-
and p53-dependent pathways, T antigen drives quiescent cells to escape p53-induced apoptosis, re-enter S-phase, and progress into cell cycle.

3. hsc70

The first 70 amino acids of SV40 Tag (J domain) bears sequence homology with the DnaJ class of molecular chaperones (Kelley and Landry, 1994), and has been shown to bind to hsc70 in host cells. The J domain is required for DNA replication and virion assembly, and during cellular transformation for the formation of dense foci (Campbell et al., 1997; Peden and Pipas, 1992; Pipas et al., 1983; Spence and Pipas, 1994; Srinivasan et al., 1997). Additionally, the J domain of Tag recruits hsc70 in order to disrupt Rb/E2F complexes and drive cell cycle entry (Srinivasan et al., 1997; Sullivan et al., 2001).

D. Immune Response to SV40 Tag T-CD8 Restricted Epitopes

The SV40 Tag oncoprotein contains in its 708 amino acid protein sequence four distinct epitopes that are recognized by SV40 specific H-2\textsuperscript{b} restricted cytotoxic T lymphocytes in C57BL/6 (B6) mice. These epitopes were originally identified using SV40-specific T-CD8 cell clones generated in H-2\textsuperscript{b} mice and syngeneic SV40-transformed cells containing various deletions in Tag, and subsequently meticulously mapped using deletion mutagenesis and overlapping synthetic peptides (Anderson et al., 1988; Campbell et al., 1983; Deckhut et al., 1992; Deckhut and Tevethia, 1992; Lippolis et al., 1995; Tanaka et al., 1988; Tevethia et al., 1984; Tevethia et al., 1990). These studies found that SV40 Tag encodes three immunodominant epitopes: epitope I (H-2D\textsuperscript{b} restricted; residues 206-215); epitope II/III (H-2D\textsuperscript{b} restricted; residues 223-231); and epitope IV (H-2K\textsuperscript{b} restricted; residues 404-411) (Figure 10).

Additionally, a single immunorecessive Tag epitope is produced from full-length Tag – epitope V (H-2D\textsuperscript{b} restricted; residues 489-497) (Figure 10), and was originally identified from Tag-specific clones (Y-5 clone) generated from B6 mice that were immunized with a Tag-variant transformed cell line in which expression of all immunodominant Tag epitopes had been eliminated (Tanaka et al., 1989). The amino acid sequence for Tag-V is encoded by residues 489-497: Gln-Gly-Ile-Asn-Asn-Leu-Asp-Asn-Leu. It was subsequently found that upon loss of this sequence, Tag-transformed variant cells were no longer susceptible to cytolysis by the Y-5 clone (Tanaka and Tevethia, 1990), confirming its specific antigenicity (Tanaka and Tevethia, 1990). Frequency analysis of SV40-Tag specific T-CD8 cell precursors in C57BL/6 mice
A. The 708 amino acid long SV40 large Tag full-length protein is represented. The four H-2^b epitopes recognized by T-CD8 are listed according to their corresponding amino acid residues (I, II/III, IV, and V) and position in the Tag dominance hierarchy. Dominant and subdominant epitopes: Tag IV >>> Tag I >> Tag II/III Tag V is designated immunorecessive, since T-CD8 cells specific for the Tag-V epitope can only be detected in the absence of other three epitopes.

B. The amino acid residues corresponding to the SV40 Tag epitopes are shown. Anchor residues of the H-2D^b-restricted Tag-V epitope - at position 5 (Asn) and position 9 (Leu) - are depicted in red.
FIGURE 10

A.

T-CD8 Cell Epitopes in SV40 Tag

<table>
<thead>
<tr>
<th>Tag Epitope</th>
<th>Dominance Hierarchy</th>
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<tr>
<td>I (206-215) H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Immunodominant (subdominant to IV)</td>
</tr>
<tr>
<td>II/III (223-231) H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Immunodominant (subdominant to IV &amp; I)</td>
</tr>
<tr>
<td>IV (404-411) H-2K&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IMMUNODOMINANT</td>
</tr>
<tr>
<td>V (489-497) H-2D&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Immunorecessive</td>
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B.

Amino Acid Sequences of SV40 Tag T-CD8 Cell Epitopes

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<tr>
<td>I:</td>
<td>Ser</td>
<td>Ala</td>
<td>Ile</td>
<td>Asn</td>
<td>Asn</td>
<td>Tyr</td>
<td>Ala</td>
<td>Gin</td>
<td>Lys</td>
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<tr>
<td>II/III:</td>
<td>Cys</td>
<td>Lys</td>
<td>Gly</td>
<td>Val</td>
<td>Asn</td>
<td>Lys</td>
<td>Glu</td>
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<td>IV:</td>
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immunized with SV40 transformed cells indicated a greater frequency of H-2K\(^b\)-restricted compared to H-2D\(^b\)-restricted precursors (Jennings et al., 1988), but only a small percentage of T-CD8 cells clones recognized Tag in association with H-2K\(^b\) molecules (Tanaka and Tevethia, 1988). The reason for this observation was explained by the finding that epitope IV, while the most immunodominant Tag epitope, is the only H-2K\(^b\)-restricted epitope (Mylin et al., 1995a; Mylin et al., 1995b).

i. **The Immunorecessive Tag Epitope V**

Exemplifying the immunodominance hierarchy of Tag in vivo is the fact that immunization of B6 mice with wild-type (WT) Tag induces T-CD8 cells specific for the three immunodominant epitopes, detectable ex vivo with MHC class I tetramers. However, no immunorecessive epitope-V specific T-CD8 cells are induced following immunization with wild type Tag-transformed cells, SV40 infection, or infection with recombinant vaccinia virus expressing full-length Tag (Fu et al., 1998; Mylin et al., 2000). It is only upon immunization with (i.) a Tag variant, in which epitopes I, II/III, and IV have been inactivated (Tanaka et al., 1989), or (ii.) with a recombinant vaccinia virus (rVV) expressing epitope-V as a minigene or within the context of a larger protein such as DHFR (Fu et al., 1998), that Tag epitope-V specific T-CD8 cells are detectable in B6 mice (Figure 11).

Although Tag-V specific T-CD8 cells are not induced following immunization with wild type Tag, the Tag-V immunorecessive epitope is efficiently presented by Tag-transformed cells in vitro (Tanaka et al., 1989). Additionally, it has been shown that the immunorecessive nature of Tag-V is maintained under conditions where Tag is exclusively cross-presented (Chen et al., 2004a). One of the potential mechanisms responsible for the immunorecessive nature of the Tag-V epitope concerns the relative instability of Tag-V/D\(^b\) complexes compared to the dominant Tag epitopes (Fu et al., 1998; Mylin et al., 1995a), especially when a fixed amount of antigen is available for cross-presentation. The presentation of cytosolic Tag-V epitope derived from a recombinant vaccinia virus encoding Tag-V (rVV-Tag V) is TAP-dependent (Otahal et al., 2005), and can be enhanced by proteasomal inhibition (Fu et al., 1998). Tag-V presentation from rVV-Tag V is also enhanced when the epitope is contained in a carrier protein construct, flanked by other Tag epitopes, as a minigene, or elongated with alanine extensions on either the amino- or carboxy- terminus of the Tag-V minimal sequence. Finally, targeting Tag-V to the ER with a Tag-V minigene preceded by an ER-directed signal sequence (rVV-ES-Tag V)
Residual T-CD8 Cell Response to T antigen in SV11 mice

A. Exemplifying the immunodominance hierarchy of Tag in vivo is the fact that immunization of B6 mice with full-length Tag induces T-CD8 cells specific for the three immunodominant epitopes – I, II/III, and IV. However, no immunorecessive epitope-V specific T-CD8 cells are induced following immunization with full-length Tag in B6 mice.

B. Upon immunization of B6 mice with individual Tag epitopes (Tag variant cells in which epitopes I, II/III, and IV have been inactivated or with recombinant vaccinia viruses (rVV) expressing individual Tag epitopes as minigenes), T-CD8 cell responses are elicited against each epitope, including Tag epitope-V.

C. In contrast, upon immunization of SV11 mice with individual Tag epitopes, no response is induced against epitopes I, II/III, or IV, as SV11 mice are tolerant of these epitopes. However, a residual T-CD8 cell response targeted against the immunorecessive Tag epitope-V is elicited.
FIGURE 11

Residual T-CD8 Cell Response to T antigen in SV11 mice

![Diagram showing immunodominant and immunorecessive epitopes]

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Immunized with</th>
<th>I</th>
<th>II/III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. B6</td>
<td>Full length Tag</td>
<td>++</td>
<td>++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>B. B6</td>
<td>individual epitope</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>C. SV11</td>
<td>individual epitope</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
dramatically augments presentation of Tag-V *in vitro* and the immunogenicity of Tag-V *in vivo* (Fu et al., 1998). These findings indicate that inadequate presentation of Tag-V is greatly influenced by the rapid cytosolic degradation of Tag-V peptides prior to TAP-mediated transport. The inefficient processing of Tag-V is improved by the initial generation of peptide fragments longer than the minimal Tag-V nonamer as well as by inhibition of peptide degradation by the proteasome and other cytosolic proteases. Furthermore, bypassing TAP with an ER targeting sequence stabilizes the level of pre-processed Tag-V peptides available for MHC class I loading in the ER, thereby enhancing presentation and immunogenicity (Fu et al., 1998).

It has recently been demonstrated that cross-presentation of the Tag-V epitope is inefficient, an effect that may stem from the intrinsic instability and short half-life of surface MHC-D\(^b\) complexes on the surface of APCs derived from a limited amount of exogenous antigen (Otahal et al., 2005). Maximal expansion of T-CD8 cells specific for Tag-V in B6 mice required cross-presentation by APCs in addition to direct presentation by immunization with tumor cells lacking expression of immunodominant epitopes, leaving Tag-V as the only expressed Tag epitope (B6/V-only Tag cells) (Figure 12B). The same study found that T-CD8 cells specific for the immunodominant Tag epitopes compete with T-CD8 cells specific for Tag-V when the epitopes are presented from the same cell. Thus, it was concluded that both poor cross-presentation and Tag-specific T-CD8 cell competition contribute to the immunorecessive nature of Tag epitope-V *in vivo* (Otahal et al., 2005).

**E. Mouse Models of SV40 Tag-induced Tumors**

The rules that govern SV40 induction of tumors in animals do not apply universally in all tissue types. A simple model suggests that cell proliferation is driven by inactivation of pRb while cell death is blocked by inhibition of p53. However, some tumors demonstrate significant apoptosis, and p53 inactivation is not always required for tumorigenesis to occur (Symonds et al., 1994; Tevethia et al., 1997). Additionally, wild type Tag yields different outcomes in transgenic mice, depending on the tissue in which it is expressed and the promoter used to drive Tag expression (Brinster et al., 1984; Campbell et al., 1983; Hanahan, 1985; Knowles et al., 1990). Thus, the interaction of large Tag with pRb and p53 tumor suppressor proteins contributes to SV40 transformation, and expression of Tag in different tissues results in
FIGURE 12

A. Adoptive Transfer of TCR Transgenic T-CD8 Cells (TCR-V cells)

In order to study the behavior of naive Tag epitope-V specific T-CD8 cells in response to Tag-expressing tumors, T-CD8 cells from TCR-V transgenic mice are used for adoptive transfer into SV11 mice with advanced tumors. TCR-V transgenic mice express the TCR α and β chains derived from a Tag epitope-V specific clone. Thus, essentially all T-CD8 cells in TCR-V mice are specific for Tag epitope-V. TCR-V mice also express the congenic marker, CD45.1 such that donor CD45.1+ TCR-V cells are distinguishable from CD45.2+ SV11 host cells. TCR-V cells isolated from secondary lymphoid organs of transgenic mice are detected with fluorescently-labeled MHC I tetramers which bind to TCRs specific for epitope Tag-V. When co-stained with mAbs to CD8 and CD45.1, the frequency of TCR-V cells in the donor population can be determined via flow cytometry. Based upon this frequency, a known number of transgenic TCR-V cells (5x10^6 cells) are injected i.v. into SV11 or B6 mice.

B. Epitope Tag-V Targeted Cellular Immunization
(B6/V-only Tag Cells)

B6/V-only Tag cells express a Tag variant in which epitopes I (residues 207-215) and II/III (residues 223-231) are deleted and epitope IV is inactivated by alanine substitution of residues 406, 408, and 411, but in which Tag epitope-V remains intact. Deleted or inactivated Tag epitopes are indicated by X. For immunization, 5x10^7 B6/V-only Tag cells are injected i.p. into SV11 or B6 mice.
FIGURE 12

A. ADOPTIVE TRANSFER of Tag Epitope V-specific $T_{\text{CD8}}$

TCR-V cells

1. CD45.1 = CONGENIC MARKER
2. TCR-V transgenic mouse
3. TCR-V cells
4. MHC class I tetramer

Adoptively transfer $5 \times 10^6$
TCR-V cells i.v.
into SV11 mice

Determine frequency of
TCR-V cell population

CD8

Tag-V Tetramer

B. Epitope Tag-V Targeted Cellular Immunization
(B6/V-only Tag Cells)

1. I
2. II/III
3. IV
4. V

B6/V-only Tag cells
histopathological outcomes that are unique to the particular cell type. Numerous transgenic mouse lineages have been created utilizing expression of Tag under various promoters in order to drive spontaneous tumor development. Depending on the anatomic and kinetic expression of Tag, immune responses to Tag are unique to each mouse line, thereby facilitating the investigation of immunotherapeutic principles in diverse tumor types.

i. **Pancreas**

The expression of the rat insulin promoter (RIP)-Tag transgene in the beta-islet cells of RIP-Tag mice induces hyperplasia and invasive solid islet tumors, progression to highly vascularized β-cell insulinomas, and enhanced production of insulin and hypoglycemia, ultimately leading to death (Christofori and Hanahan, 1994; Hanahan, 1985). Several different lineages of the RIP-Tag line have been established, and these mice differ in the kinetics of Tag expression, thus shaping the potentially responsive immune cell repertoire. RIP-Tag2 mice express Tag embryonically, and are centrally tolerant to all Tag epitopes. RIP-Tag4 mice do not express Tag in β-cells until several weeks after birth, and can be immunized to generate Tag-specific T-CD8 cells (Adams et al., 1987). If RIP-Tag4 mice are immunized with SV40 before the expression of Tag, an effective *in vivo* response is induced, which delays tumor growth for 1 year. In contrast, if immunization is administered after Tag expression, RIP-Tag4 mice cannot mount an anti-tumor response, suggesting that proper timing of immunization cooperates with kinetics of oncogene expression to maintain endogenous immunosurveillance over autochthonous tumors (Ye et al., 1994).

Given that mice of the RIP-Tag2 line are centrally tolerant to Tag, Ohashi and colleagues have crossed the RIP-Tag2 mice with the RIP-GP transgenic mouse line, which express a glycoprotein (gp33) from the lymphocytic choriomeningitis virus (LCMV-GP) (Pircher et al., 1989; Speiser et al., 1997). This enabled the investigation of tolerance and anti-tumor effect of immunotherapy in SV40 Tag induced pancreatic insulinomas that express a defined TAA not affected by central tolerance. It was found that endogenous tumor specific T-CD8 cells were not tolerized to gp33, and the persistence and responsiveness of adoptively transferred T cells was enhanced in mice with large tumor burden. Furthermore, a strong anti-tumor response was elicited following peptide immunization combined with α-CD40 agonistic mAb, or priming/boosting with viral immunization, demonstrating the potential for the combination of endogenous triggering of host APCs and vaccination to successfully modulate the immune
response to large tumors when T cells targeting the dominant epitope are present (Nguyen et al., 2002).

In the RIP-Tag4 mouse line, it was demonstrated that Tag epitope IV- but not Tag epitope I-specific T-CD8 cells are maintained long term in tumor-bearing RIP-Tag4 mice. Following adoptive transfer of large numbers of TCR-transgenic T cells specific for epitope I, these cells recognized endogenous T Ag, but were rapidly eliminated from RIP-Tag4 mice. However, immunization of RIP-Tag4 mice at 5 weeks of age against epitope IV resulted in complete protection from tumor progression over a 2-year period despite continued expression of Tag in the pancreas. This extensive control of tumor progression correlated with the persistence of functional epitope IV-specific T cells within the pancreas for the lifetime of the mice without the development of diabetes (Otahal et al., 2006). These studies demonstrated the potential for successful immunotherapy approaches targeted toward properly-timed immunization.

In the RIP-Tag5 mouse line (C3H background), it was demonstrated that administration of CpG-rich immunostimulatory oligodeoxynucleotides (CpG-ODN) induced an endogenous T-CD8 cell response, but T cells did not infiltrate tumors. However, adoptive transfer of in vitro activated T cells specific for the H-2k-restricted Tag-I epitope, coupled with immunization with Tag protein and administration of CpG-ODN rendered insulinomas permissive for T-CD8 cell infiltration and tumor destruction—an effect attributable to up-regulation of VCAM and ICAM on tumor vasculature. These data suggested that combination immunotherapy that enhances extravasation of effector cells into tumor tissue represents a critical parameter for treatment of solid tumors (Garbi et al., 2004).

ii. Bone

Line 501 mice express SV40 Tag under the influence of the α-amylase promoter, leading to Tag expression in the salivary glands and bone (Knowles et al., 1990). The expression of Tag in osteoblasts results in the development of osteogenic osteosarcomas in the bones of the axial skeleton, femur, and humerus. Frequently, tumors metastasize to the liver and occasionally to the lung. Expression of Tag is evident in the salivary glands at 3 months of age, and increases significantly by 6 months of age, without the development of neoplasia.
Osteosarcomas are detectable at 8 months, and mice live to an average of 13 months, until they succumb to tumor burden (Knowles et al., 1990).

Partial tolerance to Tag in line 501 mice appears to be epitope-dependent. Immunization of 4 month-old mice with full length Tag elicited responses against only Tag-IV, while immunization with rVVvs expressing individual Tag epitopes as minigenes elicited responses against Tag-I, Tag-IV, and Tag-V, but not Tag-II/III. Eventually, in the absence of immunization, complete tolerance developed in the periphery of 501 mice. Tolerance to Tag-I developed at 6 months and to Tag-V at 12 months of age. However, T-CD8 cells specific for Tag-IV were detectable between 3-14 months of age, unless mice had developed tumors. Additionally, lower frequencies of Tag-IV specific T-CD8 cells were seen in 3 month-old transgenic mice compared to B6 mice, and this frequency declined with age and progressive tumor growth (Schell et al., 2000).

The effect of anti-CD40 mAb administration on the priming of naive T-CD8 cells specific for Tag-I epitope against the endogenous tumor Ag in line 501 mice has been investigated. It was found that naive Tag-I-specific TCR transgenic (TCR-I) T cells underwent peripheral tolerance following adoptive transfer into 6 month-old 501 mice (Staveley-O'Carroll et al., 2003). In contrast, administration of agonistic anti-CD40 mAb led to increased expansion and acquisition of effector function by TCR-I cells, in addition to the establishment of T cell memory. The enhanced priming effect of anti-CD40 administration did not require immunization and was effective even if administered after naive TCR-I T cells had encountered the endogenous T Ag. It was concluded that anti-CD40 mAb blocked the onset of peripheral tolerance, thereby enhancing the recruitment of functionally competent effector T cells toward the endogenous Tag, implicating a pool of tumor specific cells that could potentially be harnessed for immunotherapy. The effect of TCR-I cells on ultimate control of tumor progression, however, was not investigated.

iii. **Prostate**

Expression of large and small Tag in prostatic epithelium induces hyperplasia and progression to adenocarcinomas with frequent metastasis to other tissues (Gingrich and Greenberg, 1996; Greenberg et al., 1995; Kasper et al., 1998). In TRAMP (Transgenic adenocarcinoma mouse prostate) mice, SV40 Tag is expressed under control of the rat
probasin promoter and expression is observed in the prostate and marginally in the thymus (Greenberg et al., 1995; Zheng et al., 2002). In the periphery, Tag expression is under sexual hormonal regulation, and mice remain healthy until puberty (4-5 weeks of age). After this time point, mice progressively develop prostate intraepithelial neoplasia, which progress to invasive carcinoma and metastasis (Greenberg et al., 1995; Huss et al., 2001).

It has been shown that TRAMP mice are centrally tolerant to the immunodominant Tag-IV epitope due to clonal deletion in the thymus (Zheng et al., 2002). However, while tolerance to Tag-IV, which was observed between 4-24 weeks and could not be overcome with CpG or anti-CD40 activation, tolerance was avoided following immunization with the immunorecessive Tag-V epitope (Grossmann et al., 2001). Although Zheng et al. (Zheng et al., 2002) showed that TRAMP mice were centrally tolerant to Tag-IV, one group reported that tolerance was incomplete and that early DC vaccination against Tag-IV subverted tolerance and reduced disease progression (Degl'Innocenti et al., 2005). The TRAMP model has been used to test the suitability of adoptive cellular immunotherapy for prostate cancer. Transfer of naive cells obtained from non-transgenic animals prevented development of malignant tumors, leaving minor foci of residual tumor and/or hyperplasia (Granziero et al., 1999). Adoptive transfer of memory T cells specific for Tag prevented tumor development and progression, without affecting the morphology and function of involved tissues. This study demonstrated that while normal mice possess a T cell repertoire that includes tumor specific cells that can be effectively primed against Tag, expression of the self/tumor antigen in TRAMP mice tolerizes T cells specific for Tag, thereby compromising the anti-tumor response. Recently, Anderson et al. (Anderson et al., 2007) reported that adoptive transfer of T-CD8 cells specific for Tag epitope-I followed by DC vaccination overcomes tolerance, resulting in an initial anti-tumor response. However, eventually, an immunosuppressive tumor environment quenches the T cell response, allowing for tumor outgrowth and disease progression (Anderson et al., 2007).

The studies performed in various models of Tag induced spontaneous neoplasia demonstrate the unique nature of tumors that develop progressively in distinct tissues, and the range of possible immune responses to the tumor, depending on the level, anatomic location, and kinetics of Tag expression. Such spontaneous and progressive tumor models present truer recapitulations of the scenario observed in cancer patients, and indicate the critical need to determine the state of tolerance in a tumor-bearing host. The findings from these studies demonstrate the potential success of uniquely tailored immunotherapeutic regimens guided by
knowledge gained from assessing the immune state in unmanipulated animals. Additionally, these data demonstrate similarities regarding different epitopes in multiple Tag models. Such models represent prime tools for addressing immune escape mechanisms that develop following immunotherapy and thus pose challenges for continued anti-tumor efficacy.

iv. Choroid Plexus

In other models, the introduction of SV40 early genes into the germ-line of mice results in Tag-induced tumor development in the choroid plexus (CP) – the epithelial tissue responsible for production of cerebrospinal fluid (CSF) in the brain ventricles (Brinster et al., 1984; Messing et al., 1988; Van Dyke et al., 1985). In such transgenic mice, large Tag induces hyperplastic, aggressive papillomas that are invasive and progress to carcinomas, but do not metastasize (Chen and Van Dyke, 1991). For CP tumors, the early region of Tag - which includes pRb and p107 binding regions - is essential for tumor induction, while later, more carboxy-terminal regions (p53-binding) are dispensable for tumor induction. However, deletion of p53 binding regions results in slower tumor progression (Chen et al., 1992; Saenz Robles et al., 1994).

a. The SV11 Mouse Lineage

The line SV11 mouse model of CP tumors was created utilizing a recombinant DNA plasmid (pSV11) originally constructed by Colby and Shenk (Colby and Shenk, 1982). pSV11 DNA contains full length Tag but cannot produce small tag as the splice acceptor site is deleted (Colby and Shenk, 1982). SV40 Tag is expressed as a transgene under the viral enhancer-promoter, which drives Tag expression in the thymus, renal tubular epithelium, and choroid plexus (Palmiter et al., 1985). Deletion of the 72-bp repeat enhancer region results in reduced frequency and pathology of tumors (Palmiter et al., 1985). The presence of Tag is essential for tumor induction and the transgene in inherited in Mendelian fashion with 100% penetrance (Van Dyke et al., 1987).

The SV11 line was generated from a founder animal (Palmiter et al., 1985; Van Dyke et al., 1985), and crossed for 10 generations with C57BL/6 mice, during which copy number remained constant at 10 tandem copies/haploid genome (Van Dyke et al., 1987). Both Northern blot analysis and in situ cytohybridization demonstrated high levels of p53 complexes with SV40 Tag in tumor tissue (Marks et al., 1989). Tag expression in the CP of SV11 mice is seen by 14
days after birth, and the first signs of neoplasia become evident at age 36-41 days. SV11 mice develop papillomas that grow rapidly and reproducibly until mice die at a mean of 104 days of age (Figure 13) (Van Dyke et al., 1987). SV11 choroid plexus tumors express the high-affinity \( (K_D \text{ of } 1 \text{ nM}) \) folate receptor (FR) (Roy et al., 1998), which differs from the ubiquitous low-affinity FR \( (100 \text{ µM}) \), responsible for normal folate uptake (Westerhof et al., 1991). High-affinity FRs were originally identified as tumor-associated antigens on ovarian tumor cell lines, and have more recently been described on CP tumors and ependymomas (Mantovani et al., 1994; Weitman et al., 1992).

**b. Immune Response to Tag in SV11 Mice**

SV11 mice are centrally tolerant to SV40 Tag, evidenced by the deletion of precursor T-CD8 cells specific for the immunodominant Tag epitopes (Schell et al., 1999). However, it can be postulated that the characteristics which contribute toward the immunorecessive nature of epitope V might also permit the survival of epitope V-specific T-CD8 cells in SV11 mice. This question was recently investigated, and it was found that a subset of T-CD8 cells specific for Tag epitope V are spared in SV11 mice and seed the peripheral lymphoid tissues despite Tag expression in the thymus (Schell, 2004). Despite the absence of precursors specific for the dominant epitopes, Tag epitope V maintained its immunorecessive phenotype in SV11 mice, evidenced by the failure to recruit Tag epitope V-specific T-CD8 cells upon immunization with wild-type Tag [(Schell et al., 1999) and unpublished observations] (Figure 11). Thus, it was necessary to immunize specifically against epitope V in order to detect T cells that recognize this immunorecessive epitope. It was found that the residual response to epitope V in SV11 mice is composed primarily of lower avidity T-CD8 cells, which recognize Tag epitope V peptide 1000-10,000-fold less efficiently than B6-derived T cells (Schell, 2004).

Following priming with a rVV expressing Tag-V as a minigene targeted to the ER (rVV-ES-Tag V), T-CD8 cells specific for Tag-V are undetectable with tetramer, but can be expanded in culture to a frequency 7-fold lower than T cells generated from B6 mice. Tag-V specific T-CD8 cells primed in SV11 mice also demonstrate reduced avidity (specific lysis of Tag-V expressing target cells and tetramer dissociation rate) compared to Tag-V specific T-CD8 cells generated in B6 mice. In B6 mice the majority of these cells utilized TCRVβ chains 2, 7, and 10, whereas clones generated in SV11 mice utilized TCRVβ chains 2, 4, 5, 7, and 8. However, after subsequent boosting with an SV40 Tag transformed cell line variant expressing Tag-V as
FIGURE 13

Expression of SV40 Tag and Progressive Development of Choroid Plexus Tumors in SV11 Mice

Transgenic expression of SV40 Tag in SV11 mice results in progressive development of choroid plexus tumors. Pictured are gross brain specimens, and choroid plexus sections of B6 and SV11 mice stained with haemotoxylin & eosin or stained immunohistochemically for Tag expression.

A. B6 mice exhibit normal gross brain structure and choroid plexus histology, and do not express Tag.

B. At 80 days of age, SV11 mice already demonstrate significant tumor burden, as can be detected both macroscopically and histologically at 100x magnification. Tumor growth correlates with significant choroid plexus expression of Tag.

C. By 100 days of age, at which point SV11 mice are moribund, Tag expression is detectable throughout the choroid plexus. Tumors have progressed dramatically, as can be seen by highly-vascularized masses that obliterate the brain ventricles.
Progressive Development of Spontaneous Choroid Plexus Tumors in SV11 Mice Expressing SV40 Tag

A. Gross Brain Specimen

B. Haeomotoxylin & Eosin Histology

C. Immunohistochemistry for Tag

the only Tag epitope (B6/V-only Tag cells), both the frequency (5%-13% of CD8) and avidity (% specific lysis of wild type Tag transformed cells) of Tag-V specific T-CD8 cells was enhanced, and TCRVβ chain usage was phenotypically similar to cells generated in B6 mice (skewed toward TCRVβ7 clones). These data indicated that a small number of higher avidity clones specific for the Tag epitope V are present in the endogenous repertoire of SV11 mice and these T cells remain sensitive to immunization (Schell, 2004).

c. TCR-V Transgenic Mice

A TCR transgenic mouse line, designated TCR-V mice, was created from one of the high-avidity Tag epitope-V specific T-CD8 cell clones isolated from B6 mice (Y-5 clone) (Otahal et al., 2005; Tanaka et al., 1989) (Figure 12A). TCR-V cells expressed TCRVβ7 with high penetrance (Schell, 2004). In the spleen, 18% total lymphocytes were CD8+ TCRVβ7+, and 98% of all CD8+ cells were positive for both TCRVβ7 and Tetramer-V as detected by flow cytometry. To address tolerance to the immunoressive epitope, SV11 mice were crossed with TCR-V mice. In the progeny from this cross, thymus cellularity was decreased, as well as frequency of TCR-V cells (64% of CD8+ cells) compared with TCR-V mice (97% of CD8+ cells). In the periphery (spleen), a 5-fold reduction in TCR-V cell frequency as percentage of total splenocytes was observed (3% vs 17%) and a 20-fold reduction in total number of TCR-V cells in TCR-VxSV11 mice compared to single TCR-V transgenic mice (Schell, 2004). This indicated that expression of Tag has the potential to shape the developing T-CD8 repertoire specific for Tag-V through central toleragenic mechanisms.

d. Known Effects of Immunotherapy in SV11 Mice

It has been demonstrated that H-2b-restricted T-CD8 cells specific for immunodominant Tag epitopes harbor the potential to induce control of SV11 tumors. Specifically, SV11 mice adoptively transferred with unprimed B6 cells and immunized with rVV expressing full-length or individual Tag epitopes as minigenes elicited a potent response against epitope IV which correlated with control of tumor progression (149 days and 136 days, respectively). This effect was even further enhanced when mice were irradiated prior to adoptive transfer and immunization (250 days) (Schell et al., 1999). It was also found that naive splenocytes from B6 mice are primed in SV11 mice against the endogenous Tag upon adoptive transfer and
irradiation. This treatment led to expansion of functional T-CD8 cells specific for the immunodominant Tag-IV epitope and increased lifespan to 170 days. Additionally, irradiation followed by reconstitution with immune splenocytes from B6 mice previously immunized with wild-type Tag led to even greater tumor control (277 days) (Schell and Tevethia, 2001). Finally, treatment of SV11 mice with IL-12 leads to increased survival (120-125 days), correlating with increased T cell infiltration and delayed tumor progression as observed by gadolinium-enhanced MRI analysis and reduction in tumor vascularity. However, progressive tumors expressed significant levels of TGFβ (Roy et al., 2000) and a significant percentage (15-20% tumor cells) were found to incorporate BrdU in a 2-hour pulse, indicating the aggressive nature of these tumors (Gawlick et al., 2004).

While these studies primarily focused on the response of T-CD8 cells for the immunodominant Tag epitope IV, SV11 mice do not harbor any Tag-IV reactive T cells in their repertoire due to central tolerance. Given the recent finding that residual high-avidity endogenous T-CD8 cells specific for the immunorecessive Tag-V epitope can be raised in tumor-bearing SV11 hosts (Schell, 2004) - a situation similar to that observed in cancer patients - the enticing possibility for Tag-V specific cells to respond to and exert control over SV11 choroid plexus tumors demanded further investigation.

X. Immunity and Neoplasia in the Central Nervous System (CNS)

A. Immune Privilege in the CNS

Historically, anatomical sites characterized by absent or limited responses to antigenic challenge, including the eye, gonads, placenta, and brain have been characterized as immune privileged sites (Barker and Billingham, 1977). Such organs harbor specialized microvascular beds that function to maintain tight regulation over the exchange of plasma proteins and metabolites, as well as lymphocyte adhesion (Pachter et al., 2003; Rubin and Staddon, 1999). In reference to T cells and the CNS, immune privilege is deemed to stem from (i.) the presence of the blood-brain-barrier (BBB), that impedes T cell entry to the CNS; (ii.) the absence of draining lymphatics that obviate T cell exposure to antigens localized to the CNS; (iii.) the paucity of resident APCs in the brain with the capacity to engage T cells; (iv.) low-level MHC class I and II expression on brain parenchymal cells; and (v.) suppression of immune responses by the local production of immunosuppressive factors (Becher et al., 2000; Brabb et al., 2000;
The existence of the BBB was originally proposed by Paul Erlich in order to explain the failure of systemically injected dye to penetrate the brain as readily as it penetrated other tissues (Erlich, 1960). In contrast to vasculature in other organs, CNS endothelial cells harbor low pinocytotic activity, lack transendothelial fenestrations, and are tightly joined by complex tight junctions. Additionally, the abluminal side of the endothelium is encased by a basement membrane, bone marrow-derived pericytes, and astrocyte processes. This complex cellular architecture surrounding the brain vasculature was long presumed to prevent leukocyte movement from the blood to the CNS (Abbott et al., 2006; Becher et al., 2000; Hawkins and Davis, 2005). However, as might be expected, the BBB is not an absolute barrier, since under certain conditions, the immune system must gain access to the brain in order to protect the organism. Specialized transporters on the luminal side of the BBB regulate movement of nutrients (glucose, amino acids, nucleosides, vitamins) into the CNS, and transporters on the abluminal side remove potentially toxic molecules out of the brain (Ohtsuki, 2004). According to molecular weight and charge, immunoglobulins and serum proteins are partitioned across the BBB, resulting in an inherent level of IgG present in the CSF compared to low levels of IgM and IgA (Bart et al., 2000). Furthermore, T lymphocytes are able to infiltrate the CNS – both as initiators and responders to disease – a process that cannot be attributed simply to BBB disruption (Mrass and Weninger, 2006; Ransohoff et al., 2003). Thus, it is clear that dynamic mechanisms function at the brain-vascular interface to regulate communication between the CNS and peripheral immune system, necessitating a re-evaluation of the dogmatic acceptance of the brain as a completely immune-privileged site (Carson et al., 2006).

B. Lymphatic Drainage of the CNS

Most naïve antigen-inexperienced T cells initially encounter antigen in secondary lymphoid tissues. The CNS lacks an anatomically defined traditional lymphatic system (Ransohoff et al., 2003), which historically supported the concept that the absence of a lymphatic system prevented the CNS antigens from draining to secondary lymphoid sites. However, this assumption has faced re-evaluation, based on observations that soluble CNS antigens can drain via the CSF along the perivascular and subarachnoid spaces through the cribriform plate into the lymphatics of the nasal submucosa (Csern et al., 1992; Csern and Knopf,
In contrast to the BBB, the ependymal lining of the ventricles lack tight junctions, and soluble CNS antigens within the interstitial fluid of the brain can either drain into the perivascular spaces or into the CSF of the ventricles (Figure 14) (Ransohoff et al., 2003).

It has long been recognized by physiologists that substances and cells introduced into the CNS can efflux from the subarachnoid space into blood and cervical lymph (Harling-Berg et al., 1999; Oehmichen, 1978). The immunological significance of the lymphatic pathway of drainage from the CNS was demonstrated in experiments conducted by Bradbury and colleagues, in which the efferent lymphatic duct extending from the retropharygeal nodes (deep cervical LNs) were cannulated and the recovery of radio-labeled albumin in efferent lymph was kinetically measured following infusion into the ventricles. A significant proportion of fluids draining from the brain were found to drain via the cervical lymphatic pathway with 100-200 times the concentration of venous blood (Bradbury and Cole, 1980; Bradbury et al., 1981; Bradbury and Westrop, 1983). Based on these and other experiments that traced the migration of injected dyes and tagged molecules, as well as mouse models expressing antigens specifically in the brain, it was concluded that CNS antigens drain to the cervical lymph nodes (Figure 14) (de Vos et al., 2002; Harling-Berg et al., 1999). These antigens may drain passively to the CLNs, or they may be captured and actively transported to CLNs by pericytes, macrophages, or DCs.

In accordance with the brain as an immune privileged site, Shirai reported in 1921 that mouse sarcoma tumors transplanted into the parenchyma of rat brain progressed and were not rejected (Shirai, 1921). It has been shown that immune responses generated towards cellular antigen transplanted into the brain depended on whether the antigen was placed in the cerebral cortex or ventricles. Tissue rejection was only initiated following implantation into the ventricles, suggesting that antigens that have access to the CSF (choroid plexus-derived antigens) harbor increased capacity to drain to the cervical lymphatics in order to initiate an immune response (Harling-Berg et al., 1999; Murphy, 1923).

C. The Toleragenic Microenvironment of the CNS

While the cellular organization of the BBB provides a mechanical means to maintain control over immune surveillance of the brain, the cytokine and extracellular microenvironment
A. Routes of Antigen Drainage from the Brain and T Cell Activation Against Brain-derived Antigens

Antigens from the CNS may drain directly or via brain-resident APCs to the cervical lymph nodes (CLN). Following presentation of the CNS-derived antigen either by resident DCs in the CLN or by DCs that have migrated from the brain, leads to T-CD8 cell activation, proliferation, and differentiation. Activated T-CD8 cells traffic back to the original site of antigen in the brain in order to target the CNS-derived antigen.

B. Afferent and Efferent Mechanisms of Immune Surveillance in the Brain

The choroid plexus, cerebral ventricle, subarachnoid space, brain parenchyma, systemic circulation and a peripheral lymph node are shown in cartoon form. Afferent signals from the CNS parenchyma to the peripheral immune system are initiated by movement of soluble proteins into the cerebrospinal fluid (CSF), either from white matter across the ependyma or from grey matter along the perivascular channels. From the CSF, soluble proteins are transported through lymphatic channels to peripheral lymph nodes and can provide antigenic stimulation to naive or memory T cells. The efferent phases of immune reactions are initiated in secondary lymphoid organs and promoted locally by restimulation through interactions between memory T cells and antigen-presenting cells (APCs). APCs of the CNS include a variety of myeloid-lineage cells, which give rise to many sites of potential efferent immune interaction. Resident APCs of the CNS include: choroid-plexus macrophages (a), epiplexus cells (b), meningeal macrophages (c) and perivascular cells of the Virchow–Robin spaces (d).
FIGURE 14

Routes of Antigen Drainage from the Brain and T Cell Activation Against Brain-derived Antigens

A.

B.


also participates in ensuring that robust inflammatory reactions are not a common occurrence in this anatomically protected site. In particular, TGFβ plays a significant role in establishing an immunosuppressive environment in the brain. TGFβ is produced constitutively at demonstrable levels in the CNS (Johnson et al., 1992). At its physiological concentration TGFβ has the ability to suppress proliferation of brain-infiltrating T cells (Taylor and Streilein, 1996) and down-regulate CNS vascular adhesion molecules, markedly reducing leukocyte migration across CNS endothelium (Fabry et al., 1995).

Pro-inflammatory cytokines such as TNFα are normally absent from the CNS. Exposure to TNFα induces marked changes at the BBB, leading to increased permeability and enhanced adherence and transendothelial migration of leukocytes (Fabry et al., 1995). The CNS is extremely sensitive to pro-inflammatory materials present in the blood. Injection of TNFα or IFNγ directly into the circulation leads to increased expression of adhesion molecules on CNS endothelial cells, and induces activation of cells located behind the BBB (Hickey et al., 1992).

D. Immune Cell Trafficking into the CNS

Activation of the peripheral immune system may lead to BBB leakiness caused by systemic production of inflammatory mediators such as TNFα and nitric oxide, leading to transmigration of activated macrophages and DCs across the BBB. Breakdown of the BBB marks a critical step in the development and progression of inflammation and is reflected by enhanced leakage of plasma markers into the CNS. However, this breakdown does not imply that leukocytes will gain passive access to the CNS under these conditions (Engelhardt and Ransohoff, 2005; Ransohoff et al., 2003). Rather, the local endothelium upregulates expression of adhesion molecules including E/P-selectin and VCAM-1, allowing for active recruitment of immune cells. Furthermore, in contrast to the constitutive expression on the vasculature in other anatomical sites, such molecules are not normally expressed by brain endothelium, exemplifying the strict regulation of immune surveillance by the CNS (Engelhardt, 2006a; Hickey et al., 1991).
i. Ports of Immune Cell Entry into the CNS

Three distinct routes of leukocyte entry into the CNS have been defined (Figure 14B). These are most commonly observed during inflammatory conditions and include postcapillary venules in the parenchyma, meninges, and choroid plexus (Engelhardt and Ransohoff, 2005; Ransohoff et al., 2003). Entry through the first 2 barriers is regulated by the BBB. The blood-CSF barrier differs from the BBB, as capillaries in the CP are fenestrated and lack tight junctions. Instead, tight junctions between CP epithelial cells constitute the physical barrier at this site (Engelhardt et al., 2001). It has been shown that fluorescently labeled cells demonstrated extravasation across the fenestrated CP endothelium through the epithelium directly into the CSF (Ransohoff et al., 2003). Thus, the T cell route from the blood to CSF via the CP and ventricles provides an alternative route of entry for T cells to the CNS, bypassing the BBB entirely.

a. From blood to CSF across the choroid plexus

In this immigration pathway, cells extravasate across the fenestrated endothelium of the CP stroma, migrate through the stromal core to the villi, interact with epithelial cells of the CP, and enter the CSF (Figure 14B). Support for this route stems from experiments in which fluorescently labeled cells were injected i.v. into healthy mice, and detected 2 hours later in the CP stroma, a route that was partially dependent on P-selectin (Carrithers et al., 2002). T cells comprise the main population of leukocytes in the CSF, representing 80% of CSF cells, compared with 45% of peripheral blood cells (Svenningsson et al., 1995). Migration into the CP has also shown to involve the endothelial adhesion molecules VCAM-1, PECAM, and ICAM-1 (Fabry et al., 1992; Qing et al., 2001).

b. From blood to subarachnoid space

Leukocytes transverse postcapillary venules from the internal carotid artery, across the subarachnoid and Virchow-Robbins perivascular spaces, where they potentially encounter APCs. As these zones are in direct communication with CSF, these perivascular spaces are considered putative sites of lymphocyte-APC interaction (Figure 14B) (Ransohoff et al., 2003).
c. **From blood to parenchymal space**

In this pathway, leukocytes enter the parenchyma directly from internal carotid arteries through the vascular arteriole/capillary tree and extravasate through postcapillary venules. Migrating cells are required to traverse the BBB, and while it has been shown that activated T cells can undergo this mode of migration, trafficking efficiency is quite low and independent of antigen specificity (Hickey, 1999; Lassmann et al., 1993).

ii. **T Lymphocytes in the CNS**

Previous studies indicate that T lymphocytes in the CSF compartment reflect cellular events occurring in the brain (Mor and Cohen, 1992), suggesting that analysis of the distribution and phenotype of inflammatory cells in the CSF may improve understanding of the unique immunological conditions within the CNS compartment (Svenningsson et al., 1995). Currently, there is no support for the concept that T cell blasts preferentially “home to” the CNS, and although no CNS-specific adhesion molecule has been identified, definite players in the trafficking molecule cascade have been elucidated. P-selectin has been found to participate in functional migration into the brain (Carrithers et al., 2002; Hickey et al., 1991; Pryce et al., 1997). T cells in the CSF express functional P-selectin ligands, and venules in the CP and meninges are immunoreactive for E/P-selectins, suggesting these molecules are involved in tissue entry of memory T cells to the CNS (Kivisakk et al., 2003a; Kivisakk et al., 2003b). Other candidates include $\alpha 4\beta 1$/VCAM-1 and LFA-1/ICAM-1 interactions (Qing et al., 2000; Wong et al., 1999), engagement of CD38 on T cells by endothelial PE-CAM (Engelhardt, 2006b; Qing et al., 2001), and up-regulated expression of CD44 on T cells (Piccio et al., 2002). Additionally, the role of the T cell surface glycoprotein CD43 has been implicated providing co-stimulation and promoting CD8+ T cell trafficking to the brain as well as contributing to the ultimate contraction of the immune response (Onami et al., 2002).

Chemokines may also affect leukocyte immigration into the CNS at the level of the BBB, as treatment with pertussis toxin (PTX), an inhibitor of G$\alpha$i-coupled receptors, prevented adhesion of activated T cells to endothelium and inhibited migration. Specifically, CCL2, CCL4, and CCL5 as well as the CCR7 chemokines (CCL19/CCL21) and the CXCR3 chemokine - fractalkine - appear to significantly contribute to chemoattraction of T cells into the CNS.
The labeling of T lymphocytes with fluorescent dyes, congenic markers, transgenes, or viral transduction enables T cell infiltration of the CNS to be traced (Flugel and Bradl, 2001). T cells possess the ability to pass through the CNS parenchyma in search of antigen. While the T cell frequency is low in the CNS of a healthy animal (Hickey, 1999, 2001), strong systemic immunological reactions correlate with elevated T cell numbers in the CNS (Hickey and Kimura, 1987). This indicates that if the immune system is stimulated by a specific challenge, the entire body, including the nervous system is surveyed (Lehmann, 1998).

Most data indicate that entry of T lymphocytes into the CNS requires that the T cells have been recently stimulated to blast phase, whereas naïve or resting cells do not gain CNS access (Fritz et al., 2000). The role played by T cells passing through the CNS has been characterized as antigen-seeking. It has been hypothesized that activated T cells enter the CNS in a random manner, and only demonstrate accumulation following encounter and recognition of their cognate antigen (Flugel et al., 1999; Hickey et al., 1991; Qing et al., 2000). T cells that have been specifically stimulated outside of the CNS enter the pool of recirculating cells passing through peripheral organs (Figure 14A). Hickey et al. (Hickey et al., 1991) have suggested that activated T cells of any specificity can penetrate the uninflamed BBB and that the concentration of such T cells in the CNS peaks between 9-12 hours post injection. Strikingly, only recently activated T cell blasts could enter the uninflamed CNS. Foreign antigen-reactive T cells, which were unable to find their antigen in the CNS, returned to baseline levels 24-48 hours later, while autoreactive T cells that encountered their antigen in the CNS remained, suggesting that the CNS does not appear to be exempt from general immune surveillance.

The process of T cell accumulation in the CNS has been defined in several phases (Qing et al., 2000). In Phase I (The Entry Phase) activated T cells, but not naïve or resting T cells adhere to and migrate across the BBB in a non antigen-specific manner (Hickey et al., 1991). In Phase II (The Retention Phase) antigen-specific T cells are selectively retained and may be further activated in the CNS (Hickey, 1991). This phase is highly regulated by the presence of antigen. In Phase III (The Recruitment Phase) leukocytes from the circulation are attracted to the site of initial inflammation. This phase is primarily non-antigen specific, and
involves a larger number of cells, regulated by newly expressed adhesion molecules and chemokines (Hauzenberger et al., 1995; Springer, 1994). Ultimately, the CNS microenvironment can be hostile towards infiltrating cells, and T cells in the CNS die rapidly via an apoptotic mechanism (Bauer et al., 1998). T cell death does not appear to rely upon antigen recognition, and may involve inherent CNS environmental signals such as Fas/FasL interactions and gangliosides (Hickey, 2001; Irani and Griffin, 1996; Irani et al., 1996).

iii. **APCs in the CNS**

If brain-derived antigens continuously drain to CLNs, why do foreign grafts placed into the CNS fail to elicit proinflammatory responses? The answer may lie in the nature of the APCs that present CNS-derived antigens. Cell-associated antigens are actively transported by APCs to draining nodes, and the ability to drive T cell activation is highly dependent on the activation state of the APC. It is quite possible that the DC populations in the brain that process CNS-derived antigens migrate to the CLNs, but do not express sufficient MHC and costimulatory molecules to drive T cell responses, thereby inducing tolerance instead. The two basic flavors of APC that exist in the CNS include CNS-resident paranchymal CD45lo microglia and a bone-marrow derived CD45hi population in the meninges and choroid plexus that is often replenished and includes pericytes, macrophages and DCs (Hickey and Kimura, 1988; Matsumoto and Fujiwara, 1987; Vallieres and Sawchenko, 2003).

Microglia represent brain-resident macrophages and can be identified by the expression of CD11b and low expression of CD45. In contrast, myeloid bone marrow-derived DCs found in the CNS are characterized by the expression of CD11c and are CD45hi. These DCs do not express CD8, although some may express DEC-205 (Ling et al., 2003), CD11b and F4/80 (Finn, 2003b). It was found that high numbers of blood-derived CNS-DCs were recruited following focal cortical ischemia, suggesting that brain infiltrating DCs play a role in the inflammatory response within the CNS (Reichmann et al., 2002). It has also been demonstrated that while CNS-DCs are phenotypically immature under steady-state conditions, chronic infection resulted in CNS-DC up-regulation of MHC class II, CD40, CD80/86, triggered Ag-specific T cell responses, and production of IL-12, proffering a mechanism by which DCs contribute to inflammatory responses in the brain (Fischer et al., 2000).
In specific reference to the choroid plexus tissue, a network of DCs that express MHC class II and CD11b has been identified (Matyszak and Perry, 1996; McMenamin, 1999; Vass et al., 1986). Phenotypically, CP-DCs resemble immature DCs (CD40-/CD80-/CD86-) and it has been suggested that these cells are prone to production of IL-10 under steady-state conditions, implicating such DCs intimately associated with CSF as participants in maintaining immune tolerance in the brain (Pashenkov and Link, 2002; Serot et al., 2000). DCs are also present in CSF and their frequency increases upon inflammation (Pashenkov et al., 2002; Pashenkov et al., 2003). It has been hypothesized that the vigorous immune response observed following antigen inoculation into the CSF is precipitated by rapid uptake and antigen presentation by such APC populations (Brabb et al., 2000).

Currently, no data exist on the ability of microglia or macrophages to migrate out of the CNS in significant numbers and present antigens in secondary lymphoid organs. In addition, putative microglial cells are less efficient at priming naïve T cells than DCs (Aloisi et al., 1999). CD11c+ DCs are the chief cell capable of cross-priming a CD8+ T cell, and it it possible that the cross-presentation capabilities of DCs contribute to the response against tumors and other antigens in the CNS (Aloisi et al., 1999; Calzascia et al., 2003). Fabry and colleagues have elegantly demonstrated how migration of brain-derived DCs to the CLNs may initiate immune responses. Using intracerebrally-injected fluorescent antigen, it was shown that DCs expressing CD11c and high levels of MHC class II processed antigen and accumulated at the site of injection (Ling et al., 2003). These DCs migrated from the brain to CLNs in a pertussis toxin-sensitive fashion (Karman et al., 2004b). Additionally, an antigen specific T cell response was initiated in the LNs and spleen, which subsequently drove migration of activated T cells into the brain – an effect that could not be achieved with intravenous injection of antigen-loaded DCs. These data suggested that the initiation of T cell responses to CNS-derived antigens involves emigration of brain DCs from nervous tissue to peripheral lymphoid tissues (Karman et al., 2004b; Ling et al., 2003). Thus, data acquired from multiple sources imply that primary immune responses in the CNS are initiated in a similar manner as in other organs and tissues (Karman et al., 2004a).

Much experimental data exists to suggest that DCs located in the meninges, CP, and CSF constitute a pool of DCs capable of inducing T cell responses against antigens present in the CSF space (Figure 14A). All responses appear to be generated in the CSF-draining deep cervical LNs (Harling-Berg et al., 1999; Stevenson et al., 1997). It has been observed that
APCs in the meninges, CP, and CSF can migrate to deep CLNs and efficiently activate T cells (Carson et al., 1999). When the CNS is affected by inflammation, the numbers of DCs in these locations increases significantly, most likely due to active recruitment from the circulation (Pashenkov et al., 2002; Serafini et al., 2000). DCs located in close proximity with the CSF are likely to be imprinted by molecules in the CSF, and it has been shown that cytokines such as GM-CSF, TGFβ, IL-10, and TNFα may be present in CSF of patients and that these molecules differentially affect the phenotype and functions of monocyte-derived DCs (Pashenkov et al., 2002).

E. The Choroid Plexus

i. Anatomy and Physiology

The choroid plexus tissues are papillary structures located within the brain ventricles and form the interface between the peripheral blood and cerebrospinal fluid (CSF) (Figure 15A). Choroid plexus epithelial cells actively produce and secrete the CSF, which circulates through the subarachnoid space and is ultimately resorbed into the venous blood circulation (Spector, 1977; Strazielle and Ghersi-Egea, 2000). As well as playing a significant role in maintaining intracranial pressure, CP functions include the transport of nutrients including vitamins, folate, and nucleosides; the synthesis of proteins contained in the CSF; and the clearance of catabolites (Davson, 1996; Strazielle and Ghersi-Egea, 2000). The process of CSF formation requires high blood flow rates that are successfully maintained in the CP due to low resistance of the choroidal vascular bed (Szmydynger-Chodobska et al., 1994).

Histologically, the CP consists of a simple cuboidal epithelium that surrounds a vascular bed, embedded in loose connective tissue. The epithelial layer folds into villi around a rich network of capillaries, and these fronds project into the CSF, enhancing the ventricular surface area of the plexus. In contrast to the tight parenchymal capillaries that form the anatomic basis of the blood-brain-barrier (BBB), the CP capillaries supplying each villus are large and fenestrated, with thin endothelial walls that facilitate passage into the villus stroma. The stromal connective tissue contains a dense network of DCs as well as a smaller number of resident tissue macrophages (Figure 15A and 15B) (Strazielle and Ghersi-Egea, 2000).
**FIGURE 15**

**Structure and Histology of the Choroid Plexus**

A. Schematic cross-section of 2 choroid plexus villi illustrating the main morphological and histological features of the choroidal tissue. The choroid plexus consists of numerous fronds projecting into the CSF, each frond composed of several villous processes. The outer simple cuboidal epithelium lays on a basal lamina and delimits an inner stromal core of connective and highly vascularized tissue. It derives from the adjacent ependyma lining the ventricle walls and differs from it by the presence of apical tight junctions. The epithelial cells are polarized and the apical membrane facing the CSF bears uneven borders of irregular microvilli and consistent groups of cilia. The lateral membranes of these cells display complex infoldings at their basal ends. Each villus contains a large capillary of the fenestrated type with very thin endothelial walls. The stromal connective tissue is composed of a loose network of collagen fibers, secreted by occasional elongated fibroblasts. Globular macrophages, rich in phagolysosomes, are also present in the stromal core and are distinct from the star-shaped dendritic cells. A few of the latter “squeeze” between the basal lamina and the choroid epithelium and extend processes between epithelial cells.

B. Stereomicrographs of choroid plexuses from the lateral ventricle (top panel) and fourth ventricle (bottom panel) isolated from adult rat brain. Both display an intense vascular bed. The enlargement (inset) highlights the extensive capillary network. The epithelial lining (about 10-µm-thick) can be seen on the outer surface, overlying a capillary. Scale bar: 1 mm

C. Histological formalin fixed, paraffin embedded sections of a human choroid plexus tumor, stained with hematoxylin-eosin and shown at 100X (top) and 200X (bottom) magnification.
FIGURE 15
Structure and Histology of the Choroid Plexus

A. Fenestrated capillary

B. Dendritic cells

C. Macrophage

Adapted from Strazielle, N.
Given their location at the blood/CSF/brain interface, CP cells are thought to mediate interactions between the peripheral immune system and the brain primarily through CP production of chemokines (Lacroix et al., 1998). Choroidal epithelial cells also express both MHC class I and class II molecules, and are capable of presenting foreign antigen (Hanly and Petito, 1998; Nathanson and Chun, 1989). Additionally, CP cells express the cell adhesion molecules ICAM-1, VCAM-1 and L-selectin (Tamatani et al., 1993), potentially implicated in leukocyte adhesion (Wolburg et al., 1999).

ii. Immune Microenvironment in the Choroid Plexus

As the CNS lacks traditional lymphatic vessels, the CSF is considered to be a functional equivalent of lymph for the CNS (Weller et al., 1996; Weller et al., 1992), and it has been demonstrated that CSF drains into the deep cervical lymph nodes (Knopf et al., 1995) (Cserr and Knopf, 1992; Widner et al., 1988). The choroid plexus is a rich source of macrophages and DCs - related to their close proximity to CSF space (McMenamin, 1999; McMenamin et al., 2003). It has been suggested that these cells sample CSF for presence of pathogens, tissue debris, and tumor cells and following antigen uptake, leave their environment, enter CSF, and enter the deep cervical LNs via lymphatics beneath the cribriform plate (Engelhardt and Ransohoff, 2005). It has been shown that DCs injected into the CSF space accumulate in the CLN, where they may potentially present brain-derived antigen (Figure 14A) (Hatterer et al., 2006).

iii. Choroid Plexus Papilloma

Choroid plexus papillomas (CPPs) are rare, slow-growing, histologically benign neoplasms of neuro-ectodermal origin, comprising approximately 2% to 4% of intracranial tumors in children, and 0.5% in adults. These tumors predominantly arise in the atrium of the lateral ventricle in children, and the fourth ventricle in adults (McCall et al., 2006; Weller and Ellison, 1996). Direct mechanical obstruction of CSF flow, hemorrhage, and overproduction of CSF may induce hydrocephalus, generally responsible for presenting symptoms such as headache, diplopia, and ataxia (McCall et al., 2006; Rovit et al., 1970; Schijman et al., 1990; Sharma et al., 1994; Wilkins and Rutledge, 1961). Although brain parenchymal invasion is an uncommon event, it has been documented (Levy et al., 2001). Metastasis of choroid plexus
papillomas can occur both along the neuraxis and extraneurally. The supratentorial and infratentorial compartments may become involved, as well as the spine from the cervical-medullary junction to the sacrum (McEvoy et al., 2000). Approximately 25% of choroid plexus neoplasms are malignant, and malignant degeneration from choroid plexus papilloma to carcinoma has been described (Chow et al., 1999; McCall et al., 2006).

a. **Histology and Pathology of Choroid Plexus Papillomas**

On gross pathologic examination, CPPs appear as lobulated encapsulated masses attached to the ventricular wall as an exophytic mass that protrudes into the ventricle (Figure 15B). The mass is well demarcated from the brain parenchyma, and may involve cyst formation and hemorrhage (McCall et al., 2006; Schijman et al., 1990; Sharma et al., 1994).

Microscopically, cells of choroid plexus papillomas generally demonstrate ultrastructural features of epithelial cells involved in fluid transport (Figure 15C). CPPs are featured by papillary structures morphologically resembling the normal choroid plexus that are covered by a single layer of epithelium of different heights (from columnar to flat) supported by a fibrovascular stroma. Ultrastructural features of CPPs include the maintenance of the apical-basal polarity of the neoplastic cells; the occurrence of relatively uncommon cilia and of variable numbers of surface microvilli; the demonstration of a uniform continuous basement membrane outlining the basal plasmalemma; the presence of junctional complexes connecting the cells near the apical surfaces; and the presence of large aggregates of glycogen granules. CPP cells are surprisingly regular, and mitotic figures are essentially absent. Oncocytic changes and mucin secretion may occur as uncommon histologic features (Gyure and Morrison, 2000; Levy et al., 2001; McEvoy et al., 2000; Rickert and Paulus, 2001). Immunohistochemically, CPPs are strongly and diffusely positive for the cytokeratins, vimentin, transthyretin, S-100, GFAP, and carbonic anhydrase C (Rickert and Paulus, 2001).

Expression of the high-affinity folate receptor (FR) deserves special mention. FRs mediate uptake of serum folates, which serve as carbon donors for purine and thymidine synthesis. The affinity of FRs for folate is on the order of 100 μM $K_D$, and this ubiquitous, low-affinity receptor is capable of providing for the folate requirements for normal cells (Goldman, 1971; Westerhof et al., 1991). However, some human cancers including choroid plexus papillomas, ependymomas, ovarian carcinomas, and mammary adenocarcinomas, are
characterized by the expression of a high affinity FR with a $K_D$ of approximately $1\eta M$ (Mantovani et al., 1994; Weitman et al., 1992); Westerhof, 1991 #1516). SV40-induced CP tumors in line SV11 mice express the high-affinity FR with properties similar to the human FR, including a $K_D$ of $1\eta M$ (Patrick et al., 1997), and immunohistochemical and flow cytometric analysis of FR on tumor cells has shown that all viable tumor cells were FR positive (Roy et al., 2004). Additionally, the high-affinity folate receptor has been useful in diagnosis as well as in therapeutic targeting of tumors, including SV40-induced choroid plexus papillomas in SV11 mice (Buist et al., 1993; Gawlick et al., 2004; Mezzzanica et al., 1991a; Mezzzanica et al., 1991b; Patrick et al., 1997; Ross et al., 1994; Roy et al., 1998; Roy et al., 2004).

iv. **Choroid Plexus Carcinoma**

Choroid plexus carcinoma (CPC) is one of the very few carcinomas limited almost exclusively to children, particularly those under 3 years of age. CPCs are different from CPPs in several aspects. First, carcinomas exhibit features of biological malignancy including piling of multilayered epithelium with increased pleomorphism, high mitotic activity, and necrosis. Second, CPCs demonstrate obvious invasion of the adjacent brain parenchyma tissue. Third, there is a loss of the regular papillary architecture of the tumor in the invading region, and the tumor cells arrange in solid sheets. Additionally, in contrast to CPPs, CPCs highly express Ki67, epithelial membrane antigen (EMA), and carcinoembryonic antigen (CEA) (Ironside JW, 2002; Rickert and Paulus, 2001; Weller and Ellison, 1996).

v. **Diagnosis of Choroid Plexus Tumors**

Choroid plexus tumors are diagnosed with either Computed Tomography (CT) scan or Magnetic Resonance Imaging (MRI). CT evaluation of CPPs demonstrates a homogeneously hypodense to slightly hyperdense enhancing mass with or without cyst area and secondary hydrocephalus. High density on CT may represent calcification or blood within the tumor. Homogeneous enhancement on contrast-enhanced CT is typical due to the marked vascularity of these tumors. The appearance of a CPP on MRI is similar to that of a CT scan and shows intermediate-to-strong intensity on both T1- and T2-weighted images (McCall et al., 2006; Rickert and Paulus, 2001; Weller and Ellison, 1996).
vi. Treatment and Prognosis for Choroid Plexus Tumors

CPPs can be cured by total surgical resection. In a recent series by McEvoy et al. (McEvoy et al., 2000), the 5-year survival rate was 100%, and tumors did not recur in half of the patients who underwent subtotal resection. CPPs with a benign cellular appearance but with evidence of local parenchymal invasion still respond to surgical therapy alone, without the need for adjuvant treatment (McEvoy et al., 2000; Rickert and Paulus, 2001). There is no consensus on the most effective treatment for choroid plexus papilloma metastases, as surgical resection, chemotherapy, and radiation therapy offer potential benefits. Finally, the prognosis for patients with disseminated choroid plexus papilloma range from stable disease and symptoms to death within months (McCall et al., 2006; Weller and Ellison, 1996).
CHAPTER III
MATERIALS AND METHODS

Mice
C57BL/6 (H-2^b^) mice, B6.SJL-H2 (H-2^b^) mice, and UBI-GFP/BL6 mice were purchased from the Jackson Laboratories (Bar Harbor, Maine) and maintained at the animal facility of the Milton S. Hershey Medical Center. SV11 mice on the C57BL/6 background express full length SV40 Tag under the control of the endogenous SV40 promoter/enhancer (Palmiter et al., 1985). The SV11 line has been maintained in the animal facility of the Milton S. Hershey Medical Center by backcrossing Tag transgene positive males with C57BL/6 females for 54 generations. SV11 transgene positive mice were identified by PCR amplification of the transgene as previously described (Schell et al., 1999). Transgene positive (SV11) and transgene negative littermates (B6) were used at 85 days of age. Line 459 mice expressing the TCR\(\alpha\) and TCR\(\beta\) chains specific for Tag epitope V on the C57BL/6 background were generously provided by Satvir S. Tevethia (The Pennsylvania State University College of Medicine, Hershey, PA) and have been previously described (Otahal et al., 2005). Line 459 mice were bred with B6.SJL-H2 mice in order to generate the TCR-V/CD45.1 congenic mouse strain, hereafter referred to as heterozygous (CD45.1/CD45.2) or homozygous (CD45.1/CD45.1) TCR-V mice, and were screened to determine transgene expression in CD8+ cells in peripheral blood. UBI-GFP/BL6 mice express the Green Fluorescent Protein (GFP) from the human ubiquitin C promoter (Schaefer et al., 2001). TCR-V mice were bred with UBI-GFP/BL6 mice in order to generate TCR-V/UBI-GFP mice. All experimental protocols were performed in accordance with guidelines established by the institutional animal care and use committee of the Pennsylvania State University College of Medicine and comply with federal guidelines.

Cell lines and media
B6/T116A1 cells (B6/V-only Tag) express a Tag variant in which epitopes I (residues 207-215) and II/III (residues 223-231) are deleted and epitope IV is inactivated by alanine substitution of residues 406, 408, and 411, but in which epitope V remains intact (Mylin et al., 2000). The cell line B6/122B1 (Tag epitope-null) expresses a Tag derivative in which all four T\(\text{CD8}\) epitopes (I, II/III, IV, and V) were inactivated by substitution of critical MHC class I anchor residues (N210A, N227A, F408A, and N493A) (Mylin et al., 2000). TAP\(^{1}\)/V-only Tag cells were generated by transfection of B6.129S2-Tap1\(^{1}\)Arp mouse primary kidney cells with plasmid pSLM361-11
(Mylin et al., 2000) encoding epitope V-only T Ag (Otahal et al., 2005). Cell lines were maintained in DMEM, supplemented with 100 U/mL of penicillin, 100 mg/mL of streptomycin, 100 mg/mL of kanamycin, 2 mM L-glutamine, 10 mM HEPES, 0.075% (wt/vol) NaHCO₃, and 10% fetal bovine serum (FBS). Ex vivo lymphocytes were maintained in complete RPMI 1640 medium supplemented with 10% FBS, 100u/mL of penicillin, 100 mg/mL of streptomycin, 100 ug/mL streptomycin, 2mM L-glutamine, and 50 uM 2-mercaptoethenol.

**Synthetic peptides**

Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by Fmoc chemistry using an automated peptide synthesizer (9050 MiliGen PepSynthesizer; Milipore, Bedford, MA). Peptides were solubilized in DMSO and diluted to 5 mM in RPMI-1640 medium. Peptides used for the intracellular cytokine assay corresponded to the SV40 Tag epitope V (QGINNLDNL; peptide V) and influenza virus nucleoprotein 366-374 (ASNENMETM; peptide Flu) (Falk et al., 1991b).

**Adoptive transfer and immunization protocols**

For adoptive transfer, RBC-depleted lymphocytes derived from spleens and LNs of TCR-V/CD45.1 transgenic mice were resuspended in HBSS, filtered, and injected intravenously in 0.4 mL into the tail vein of SV11 or B6 transgene-negative littermates at a dose of 5x10⁶ clonotypic TCR-V cells/mouse. For the in vivo cytotoxicity assays, TCR-V/CD45.2 mice were used as donors in order to identify CD45.1+ target cells. For immunization, B6/V-only Tag cells were resuspended in HBSS and injected in 0.5 mL via the intraperitoneal route at a dose of 5x10⁷ B6/V-only Tag cells/mouse. Primary and booster immunizations were administered as indicated for individual experiments.

**αCD40 Pre-Conditioning**

85 day-old SV11 or B6 mice received 100 μg of purified agonistic anti-CD40 mAb FGK45 (generous gift of Dr. Stephen Schoenberger, San Diego, CA) or polyclonal rat IgG (Sigma-Aldrich) by i.p. injection in 200 μl PBS the day prior to and the day after adoptive transfer of TCR-V cells.
Combinatorial immunotherapy protocol

85 day-old SV11 or B6 mice received 100 µg of purified agonistic anti-CD40 mAb FGK45 (generous gift of Dr. Stephen Schoenberger, San Diego, CA) or polyclonal rat IgG (Sigma-Aldrich) by i.p. injection in 200 µl PBS the day prior to and the day after adoptive transfer of TCR-V cells. Mice received 5x10^6 TCR-V cells in 400 µl via tail vein injection on the day in between anti-CD40 mAb administration. Seven days later mice were immunized with 5x10^7 B6/V-only Tag cells via intraperitoneal injection.

Lymphocyte isolation

Mice were anesthetized via i.p. injection of sodium pentobarbital (70 mg/kg body weight) diluted in 10% ethanol and perfused transcardially with PBS. Spleens, brains, cervical lymph nodes (CLN) and inguinal lymph nodes (ILN) were dissected and transferred to cold RPMI-1640. Spleens and LN were processed to single cell suspensions and spleens were depleted of RBCs using Tris NH_4Cl. To isolate lymphocytes from brains, brains were minced in complete RPMI 1640 using a razor blade, and single cells were dissociated from larger tissue fragments by repeated pipetting. After allowing debris to settle, the supernatant containing cells was collected, and the remaining clumps were disrupted by pipetting in fresh RPMI 1640. The supernatants containing cells were combined and the cells were pelleted by centrifugation. Cells were resuspended in 3 mL 70% Percoll (Sigma, St. Louis, MO) in 15-mL conical tubes. 5 mL of 30% Percoll was layered on top of the cells, and the gradients were centrifuged at 500 x g for 25 minutes at 4°C. Cells were harvested from the gradient interphase and washed once with complete RPMI 1640 before use.

Isolation of tumor and stromal cells

Mice were anesthetized via i.p. injection of sodium pentobarbital (70 mg/kg body weight) diluted in 10% ethanol and perfused transcardially with PBS. Brains were dissected and transferred to cold RPMI-1640. Using a razor blade, tumors from SV11 brains were dissected from remaining brain tissue. Brains of B6 mice and tumors of SV11 mice were minced in complete RPMI 1640 using a razor blade, and single cells were dissociated from larger tissue fragments by repeated pipetting. Minced brains and tumors were washed in RPMI-1640 and resuspended in 10 mL of 1 mg/mL Type II collagenase (Worthington Biochemicals) diluted in RPMI-1640 and continuously rocked for 20 minutes at 37°C. 10 mL of 0.1M EDTA was added to stop the enzymatic reaction, followed by washing with 10 mL of RPMI-1640 three times.
Resulting cells were stained for flow cytometric analysis. For detection of choroid plexus tumor cells expressing the high-affinity folate receptor (FR), cells were blocked with 10% normal goat serum for 20 minutes prior to primary staining with polyclonal purified rabbit α-FR Ab (1:500) (generous gift of Dr. Edward Roy, Urbana, Illinois) for 45 minutes on ice. Cells were washed 3X and stained with secondary PE-conjugated goat α-rabbit Ab (1:500) (Molecular Probes) for 20 minutes, washed 3X and resuspended for flow cytometric analysis. In addition to the α-FR stain, cells were co-stained with biotinylated α-B7-H1 mAb (1:100 dilution - primary stain) followed by streptavidin-Alexa 647 (Molecular Probes) (1:500 dilution – secondary stain); and FITC-conjugated α-CD45.2.

MHC Tetramers and antibodies
MHC class I tetrameric complexes corresponding to the H-2D\(^b\)/Tag epitope V (D\(^b\)/V) and H-2D\(^b\)/influenza virus NP epitope 366-374 (D\(^b\)/Flu) conjugated with streptavidin-PE were prepared as previously described (Mylin et al., 2000). Purified anti-CD16/CD32 was purchased from BD-Pharmingen. The following antibodies were purchased from eBioscience: PE-Cy5-labeled anti-mouse CD8a (clone 53-6-7); FITC-labeled anti-mouse CD44 (clone IM7), FITC-labeled anti-mouse L-selectin (clone MEL-14), FITC-labeled anti-mouse CD69 (clone H1.2F3), FITC-labeled anti-mouse CD40 (clone 1C10), FITC-labeled CD122 (clone TM-B1), FITC-labeled CD127 (clone A7R34), FITC-labeled CD38 (clone 90), FITC-labeled anti-CD5 (clone 53-7.3), FITC-labeled anti-mouse CD45.1 (clone A20), biotin-labeled anti-mouse CD45.1 (clone A20), PE-labeled anti-mouse CD45.2 (clone 104), FITC-labeled anti-mouse CD11c (clone N418), biotinylated anti-mouse CD11c (clone N418), PE-labeled anti-mouse CD11b (clone M1/70), APC-labeled anti-mouse CD44 (clone IM7), APC-labeled anti-mouse L-selectin (clone MEL-14), PercP-Cy5 labeled anti-mouse CD8a (clone 53-6-7), biotinylated B7-H1 (M1H5), FITC-labeled anti-BrdU (clone PRB-1), FITC-labeled anti-mouse PD-1 (clone J43); APC-labeled anti-mouse F4/80 (clone BM8), FITC-labeled anti-mouse Ly6C (clone AL-21), PE-labeled anti-mouse TCR\(\beta\) (clone H57-597), PE-Cy5-labeled anti-mouse Gr-1 (clone RB6-8C5), functional grade anti-mouse B7-H1 (clone M1H5), functional grade anti-CD3 (clone 145.2C11), PE-conjugated anti-mouse TNF\(\alpha\) (clone MP6XT22), FITC-conjugated anti-mouse IFN\(\gamma\) (clone XMG1.2), PE-conjugated anti-mouse IL-4 (clone 11B11), APC-conjugated anti-mouse IL-10 (clone JE55-16E3), FITC-labeled anti-mouse H-2D\(^b\) (clone KH95), FITC-labeled anti-mouse H-2K\(^b\) (clone AF6.88.5). Unlabeled streptavidin, PE-conjugated goat α-rabbit IgG, and streptavidin-Alexa-647 were purchased from Molecular Probes.
Flow cytometric analysis

For quantitative *ex vivo* characterization of isolated T cells, lymphocytes were resuspended at 2x10^7/ml in FACS buffer (PBS containing 2%FBS/0.01% NaN₃), and incubated in the presence of anti-CD16/CD32 and unconjugated streptavidin for 30 minutes on ice. Cells were washed in FACS buffer and resuspended in a cocktail of the indicated fluochrome-conjugated antibody and tetramer. PE-conjugated tetramers were diluted 1:200; FITC-conjugated mAbs (1:50); all other mAbs were diluted 1:100. Cells were stained in the dark on ice for 1 hour. In the case of biotin-conjugated mAb-containing cocktails, cells were washed 3 times in FACS buffer and resuspended in streptavidin-conjugated Alexa 647 (Molecular Probes) at 1:500 dilution for 30 minutes on ice. Cells were washed 3 times in FACS buffer, and fixed with 2% paraformaldehyde/PBS and analyzed using a FACScan or FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). Routinely 50,000 events were recorded. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

*In vivo* proliferation assay (CFSE)

RBC-depleted lymphocytes derived from spleens and LNs of TCR-V transgenic mice were resuspended at 1x10^7/mL in PBS/0.2% BSA and labeled with 5 mM CFSE (Molecular Probes) for 10 minutes at 37°C. Cells were washed three times with PBS/0.2% BSA, resuspended in HBSS, filtered through a cell strainer (Falcon), and injected i.v. at a dose of 5x10^6 clonotypic TCR-V cells per mouse in 0.4 ml. After 2, 4, or 7 days the dilution of CFSE was determined by tetramer staining of splenic, LN, and brain-derived lymphocytes (APPENDIX A).

Intracellular cytokine assay

For staining of intracellular IFN-γ, lymphocytes were isolated from the spleen, CLN, and brain, processed, and incubated in 0.2 mL RPMI-1640/10% FBS in U-bottom 96-well plates with the indicated peptides (peptide Tag V or peptide Flu) plus 1µg/mL brefeldin A (BFA) (Sigma) for 6 hours at 37°C, 5% CO₂. In some experiments, cells were incubated in wells with agonistic αCD3 mAb (10µg/mL), or PMA (2 ng/mL) + ionomycin (0.5 uM) plus 1µg/mL BFA. CD8+ T cells were stained for intracellular cytokines using the Cytofix/Cytoperm Kit (Pharmingen) in accordance with manufacturer’s specifications. Stimulated cells were washed twice and Fc receptors blocked by incubation with rat anti-mouse CD16/CD32 for 20 min, followed by staining with PE-Cy5-labeled rat anti-mouse CD8α (clone 53-6-7) (eBioscience) for 30 min. After fixation and permeabilization for 20 min, cells were stained with FITC-labeled rat anti-mouse IFNγ (BD Pharmingen) alone or isotype control or various combinations of FITC-labeled rat anti-
mouse IFNγ; PE-labeled rat anti-mouse TNFα; PE-labeled rat anti-mouse IL-4; and APC-labeled rat anti-mouse IL-10 (BD pharmingen) for 30 min and then analyzed by flow cytometry as described above. The percentage of cells that stained specifically for intracellular cytokines following stimulation with peptide V, αCD3, or PMA/ionomycin was determined by subtracting the percentage of CD8+ cells which stained for cytokine in the presence of peptide Flu-NP (APPENDIX B).

**In vivo cytotoxicity assay**

In vivo cytotoxicity assays were performed as described previously (Otahal et al., 2005). In brief, target cells were prepared from sex-matched B6.SJL (CD45.1+) spleen cells by incubation in the presence of 1 µM of the indicated peptides (peptide Tag V or peptide Flu) in RPMI/10% FBS at 37°C for 90 minutes and then washed three times to remove excess peptide. Targets were differentially labeled with CFSE (5 µM for peptide V; 0.5 µM for peptide Flu) for 10 minutes at 37°C in PBS/0.1% BSA and washed 3 times. A total of 5x10⁶ target cells (2.5x10⁶ of each) was injected i.v. into the tail vein in 0.4 mL HBSS. The elimination of CD45.1+ CFSE-labeled targets was assessed the next day (APPENDIX C). The following formula was used to determine the percentage of specific killing: % lysis = [1 - (ratio unprimed/ratio primed) x 100], where ratio = (% of CFSE<sup>low</sup> cells/% of CFSE<sup>high</sup> cells).

**In vivo BrdU labeling**

To assess in vivo proliferation of adoptively transferred TCR-V cells, mice were injected i.p. every 12 hours with 4 doses of 1 mg/mL BrdU (Sigma) diluted in PBS two days prior to sacrifice in order to label proliferating cells. Mice were sacrificed 12 hours after the fourth dose of BrdU and lymphocytes from the brain, spleen, and CLN were prepared as previously described above for lymphocyte isolation. Single cell lymphocyte suspensions were surface stained for Tag-V Tetramer and CD8 in 96 well plates as previously described above and cells were fixed and permeabilized in 200 uL 1% paraformaldehyde/0.01% Tween-20 (Sigma) overnight at 4°C. The following day, cells were washed and incubated in 200 uL bovine pancreatic DNAse I (50 Kunitz Units/mL = 0.1 mg/mL) (Sigma) prepared in PBS/Ca<sup>++</sup>/Mg<sup>++</sup> for 45 minutes at 37°C. Cells were washed twice and resuspended in 150 ul 0.5% Tween-20 diluted in PBS supplemented with 10% FBS. 20 ul of FITC-conjugated BrdU mAb (or isotype control Ab) was added to each well and stained for 45 min. Cells were washed twice and resuspended for flow cytometric analysis.
**In vitro proliferation assay**

Lymphocyte single cell suspensions from spleens and brains were prepared and separate mouse samples were stained for Tag-V Tetramer and CD8 as previously described to assess consistent frequency of Tet-V+CD8+ cells. Cells from indicated numbers of mice were pooled, labeled with 5 uM CFSE as described above, and cultured for 6 days *in vitro* in 96 well plates. All wells contained 1x10^5 brain-isolated or spleen-isolated cells and 1x10^5 irradiated B6 splenocytes as feeder cells. Some wells contained 10 U/mL IL-2 as indicated per experiment. Varying conditions included 5µM Tag-V peptide; 2 ng/mL phorbol myristate acetate (PMA) (Sigma) + 0.5 µM ionomycin (Sigma); and plate-bound α-CD3 (10 µg/mL) (clone 145.2C11). Some wells also included functional grade αB7-H1 mAb (10 µg/mL) (clone M1H5), in order to block PD-1 signaling. *In vitro* cultures were assessed on day2, 4, and 6 to detect TCR-V cell proliferation by staining for Tetramer-V, CD8, and observing dilution of CFSE (APPENDIX D).

**Apoptosis Assays**

TCR-V cells were isolated from SV11 or B6 spleens and brains at 125 days of age following adoptive transfer + prime and boost immunization. Cells were surface-stained for tetramer Tag-V and CD8. For Caspase-3 assay, cells were fixed and permeabilized in 200 uL 1% paraformaldehyde/0.01% Tween-20 (Sigma) for one hour on ice. Cells were washed twice and resuspended in 150 ul 0.5% Tween-20 diluted in PBS supplemented with 10% FBS. 20 ul of cleaved Caspase-3 (Asp175) (5A1) Rabbit mAb (Cell Signaling) or isotype control Ab was added to each well and stained for 45 minutes on ice. Cells were washed twice and stained with Alexa 647-conjugated goat anti-rabbit Ab for 30 minutes. Cells were washed twice and resuspended for flow cytometric analysis. For Annexin-V assay, surface staining was performed as follows: PerCP-conjugated anti-CD8, biotin-conjugated anti-CD45.1 followed by Streptavidin-Alexa 647, and FITC-conjugated anti-Annexin-V Ab (BD Pharmingen) for 45 minutes on ice, washed twice, and resuspended for 15 minutes in 0.1 mg/mL Propidium Iodide (Molecular Probes) diluted in FACS buffer in order to distinguish dead cells. Cells were then analyzed via flow cytometric analysis. For TUNEL assay, following surface-staining, the In Situ Cell Death Detection Kit - Fluorescein (Roche) was used and procedures followed according to manufacturer specifications. Briefly, cells were fixed in 2% paraformaldehyde for 1 hour at room temperature and washed twice. Cells were fixed for 2 mins in permeabilization buffer (10% Triton-X/0.1% Sodium citrate in PBS) and washed twice. 200 ul of Dnase I (10,000 U/mL) (Sigma) was added to each well for 10 mins at room temperature and washed twice. TUNEL reaction mixture (Roche) was prepared and 50 uL added/well for 1 hour at 37°C. Cells were
wshed twice and resuspended for flow cytometric analysis. As a positive control for apoptosis, cells were treated with 5 µM camptothecin overnight at 37°C.

Immunohistochemistry
Brains were harvested from perfused mice as described above, imbedded in optimal temperature compound (Tissue-Tek, Sakura Finetek, Inc.), snap-frozen in liquid nitrogen and placed at -80°C. Ten µm sections were cut using a cryostat (Bright OTF, Hacker Instruments), mounted on slides, fixed in acetone at -20°C for 10 minutes and air-dried at room temperature. Endogenous peroxidase was quenched by incubation of sections in 0.03% H2O2/PBS for 15 minutes at room temperature followed by washing. Sections were blocked with normal goat serum (Histomark) for 30 min and stained with primary biotinylated rat anti-mouse CD8a mAb (clone 53-6.7, Pharmingen) at room temperature in a humidified chamber for 1 hour. Sections were washed twice in PBS and stained with biotinylated goat anti-rat IgG (H+L) secondary mAb (Histomark) for 1 hour. Controls were stained with secondary mAb only. Slides were washed twice in PBS, incubated in streptavidin-peroxidase (ChemMate Kit; Ventana, Tuscon, AZ) for 30 minutes and washed in PBS. The chromagen diaminobenzidine (DAB, ChemMate) was added for 2-10 minutes, and slides were washed in PBS followed by dH2O. Finally, sections were mounted in Crystal/Mount (Biomeda, Foster City, CA) and examined using a Nikon Microphot-FXA microscope. Representative images were captured using a Sony DKC-ST5 color digital camera.

H&E Histology
Mice were anesthetized via i.p. injection of sodium pentobarbital (70 mg/kg body weight) diluted in 10% ethanol and perfused transcardially with PBS, followed by pericardial transfusion with 10 mL 4% paraformaldehyde. Brain were dissected and fixed in 4% paraformaldehyde for at least 24 hours prior embedding in paraffin blocks. Sections were cut and collected onto slides and stained with hematoxylin and eosin. Sections were examined using a Nikon Microphot-FXA microscope and representative images were captured using a Sony DKC-ST5 color digital camera.

Immunofluorescent imaging and confocal microscopy
Freshly isolated brains from perfused mice were harvested and cut coronally in half. One half was used for flow cytometric analysis, and the other half was fixed overnight at -20°C in 4% PBS/paraformaldehyde (PF), followed by imbedding in optimal temperature compound. Brains
were snap-frozen in liquid N\textsubscript{2}, cut into 10 um sections and mounted on slides. Sections were fixed in acetone at -20°C for 10 minutes and air-dried at room temperature. Slides were washed 2 times in PBS, blocked in 10% normal goat serum for 30 minutes, and stained with primary mAb rat anti-mouse CD8a (clone 53-6.7) at 1:500 for 1 hour in a humidified chamber at room temperature. Slides were washed 3 times in cold PBS and stained with secondary Alexa 647-conjugated goat anti-rat IgG (H+L) mAb (Molecular Probes) diluted 1:500 for 1 hour in a humidified chamber at room temperature. Slides were washed 3 times in cold PBS, counterstained in DAPI for 5 minutes, mounted in Aqua Poly/Mount (Polysciences; Warrington, PA) and coverslipped. Visualization of GFP-positive cells required no additional antibody staining but could be visualized following the post-fixation step using fluorescence microscopy. Fluorescence analysis was performed with a confocal laser-scanning microscope (TCS SP2 AOBS; Leica, Deerfield, IL) at 512 x 512-pixel resolution. Images were maximum projections of z-stacks. The brightness and contrast of some images were adjusted with image-analysis software (Photoshop; Adobe, Mountain View, CA) and the digital resolution was held constant.

**Lifespan analysis**

SV11 mice were monitored for the development of hydrocephalus, indicative of end-stage choroid plexus tumors. Mice were euthanized following the development of neurological symptoms. The presence of tumors was confirmed by gross examination. In some cases, spleens and brains were processed and stained for flow cytometric detection of TCR-V cells. Survival curves were constructed by the Kaplan-Meier method with GrafPad Prism software (GrafPad Software, Inc. San Diego, CA). Significance was determined by single-factor ANOVA, and validated using the log-rank test. $p$ values of <0.05 were considered significant.

**Magnetic Resonance Imaging (MRI)**

MRI studies were conducted in the PSU Center for NMR Research (CNMRR) in accordance with all guidelines established for conducting research in the CNMRR as reviewed by the Protocol Review Committee (PRC). Mice were transported to the PSU Center for NMR Research MRI facility in filter top cages that were placed within a thermally protective container. MRI was performed on a 7.0 T Bruker Biospin system using a 3 cm birdcage coil, one day prior to TCR-V cell adoptive transfer, and then at 20 and 40 days following adoptive transfer and administration of combinatorial immunotherapy. Prior to imaging animals were anesthetized with isoflurane (4% induction, maintenance with 1.5%). Anesthesia was maintained during all MRI studies with 1.5% isoflurane in compressed air flow (2-3 l/min) through a mask fixed to the
stereotactic frame via a tubing (about 20 yards) from the vaporizer. During the imaging rectal body temperature and respiration of the mice were monitored and maintained with a MR-compatible Small Animal Monitoring & Gating System (SA instrument, Inc, Stony Brock, NY) Anesthesia levels were adjusted if breathing was reduced below 20 BPM, or was above 40 BPM. Heated air was used to maintain mouse's body temperature between 35-37 °C during the imaging. Mice received an intravenous injection of 25 nmol of gadoversetamide contrast reagent (Magnevist, Berlex Laboratories, Wayne, NJ) immediately prior to analysis. A pilot scan was first performed to allow for consistent positioning of the slices of interest in each mouse. T1-weighted spin echo (15 slices, 1 mm slice thickness, TR/TE=500/8.2 ms, 156X156 µm² resolution and 8 averages) images were acquired, with each scan lasting approximately 45 minutes. After imaging each mouse was recovered on a heating pad. Mice were returned to their cages after analysis and transported back to the central animal facility. Post-contrast hyperintense volumes in T1-WI images were determined using an automated segmentation routine, CCHIPS. For MRI data analysis, tumor volumes before and after treatment were analyzed using ANOVA.
CHAPTER IV

CD8+ T CELLS SPECIFIC FOR AN IMMUNORECESSIVE EPITOPE RECOGNIZE ENDogenous T ANTIGEN IN SV11 MICE, ACCUMULATE AT THE TUMOR SITE, AND CONTROL DISEASE PROGRESSION FOLLOWING SECONDARY IMMUNIZATION

GOALS
The hypothesis driving these studies proposed that within the context of a spontaneous tumor, immunotherapeutic strategies that target T-CD8 cells specific for an immunorecessive epitope, could be recruited for the control of cancer progression. To this end, the purpose of Specific Aim I was to monitor the effect of endogenous Tag on the fate of immunorecessive Tag epitope V-specific (TCR-V) T-CD8 cells within the tumor-bearing host environment, and determine the potential for TCR-V cells to control tumor growth. The response of TCR-V cells to the endogenous Tag as well to Tag-V specific cellular immunization was explored. This was accomplished by monitoring TCR-V cells for activation, proliferation, acquisition of effector function, tumor infiltration, in vivo persistence, and response to tumor growth following adoptive transfer into SV11 mice.

OVERVIEW
The results demonstrate that TCR-V cells recognize endogenous Tag - indicated by proliferation and acquisition of effector function in vivo - but fail to accumulate and do not traffic to the tumor. Secondary immunization specifically directed toward Tag epitope V promoted accumulation of transferred TCR-V cells at the tumor site and enhanced SV11 survival. It was concluded that while immunorecessive Tag epitope V-specific TCR-V cells recognized endogenous Tag in SV11 mice, immunization targeted against Tag epitope V was necessary to promote accumulation of TCR-V cells at the tumor site, allowing for modest control over tumor progression. However, ultimate morbidity and mortality of SV11 mice correlated with a failure to maintain TCR-V cells at the tumor site – an observation that can partially be explained by an increased susceptibility of TCR-V cells to apoptosis.
INTRODUCTION

Tumor responsive T-CD8 cells present in the endogenous repertoire have often been targeted for immunotherapy of antigenic tumors (Melief et al., 2000b). Numerous studies have documented the in vivo capacity of T-CD8 cells to recognize and eliminate tumors in experimental models (Greenberg, 1991; Melief et al., 2000b). Most tumor-associated antigens that have been identified in cancer patients are self-antigens, derived from non-mutated cellular proteins (Boon and van der Bruggen, 1996). However, generation of the T cell repertoire normally includes the elimination of potentially self-reactive thymocytes during the process of negative selection (Stockinger, 1999). Thus, the T-CD8 cell population exported from the thymus is composed primarily of cells that demonstrate low-avidity for thymic-expressed self antigens or higher avidity clones that recognize tumor-associated self-antigens that are either (i) not expressed in the thymus (Boon and van der Bruggen, 1996), or (ii) expressed at low levels (Stockinger, 1999). Consequently, the peripheral T cell repertoire has usually been purged of T-CD8 cells with the most potent anti-tumor reactivities (Ashton-Rickardt et al., 1994). Therefore, investigations that seek to maximize the responsiveness of tumor-specific T-CD8 cells must account for the limitations imposed by the mechanisms that maintain self-tolerance. The ability to specifically recruit T-CD8 cells within the tumor-bearing host environment provides possibilities for the strategic design of immunotherapeutic approaches to cancer.

Expression of tumor antigens as a self-antigen has been shown to alter the immunodominance hierarchy of T-CD8 cells reactive towards the tumor. Experimental evidence suggests that T-CD8 cells specific for subdominant or immunorecessive self/tumor antigens are less susceptible to tolerogenic mechanisms, thereby permitting the persistence of both low and high avidity epitope-specific T cells despite the continuous presence of antigen (Friedman et al., 2004; Lotz et al., 2004; Theobald et al., 1997). A few studies have reported that immunization against subdominant tumor epitopes resulted in protection from transplantable tumor challenge in the absence of self antigen expression (Duraiswamy et al., 2004; Feltkamp et al., 1995; Newmaster et al., 1998) or revealed that T cells specific for an immunorecessive epitope can be recruited in the context of self antigen expression (Grossmann et al., 2001; Schell, 2004; Schell et al., 2000). However, the ability of these T-CD8 cells to control the progression of spontaneous tumors was not addressed.

Mice that develop spontaneous tumors due to the transgenic expression of an
oncoprotein provide realistic models to study the recruitment of T-CD8 cells against a self/tumor antigen. The SV11 mouse provides an ideal model for the study of the immune response to spontaneous cancer, as the tumor-specific T-CD8 cell repertoire has been tolerized to the self-tumor antigen, resulting in deletion of T-CD8 cells that recognize dominant Tag epitopes. Thus, the SV11 model recapitulates characteristics of the tumor-reactive T cell repertoires often observed in the clinical scenario. Additionally, the well-characterized T-CD8 cell response to SV40 Tag allows for in-depth analysis of the T-CD8 cell response to rapidly-progressing SV11 choroid plexus tumors, particularly T-CD8 cells specific for the immunorecessive Tag epitope V clones - representative of the sole residual Tag-reactive T-CD8 cell population in SV11 mice.

The low frequency of Tag-V-specific T-CD8 cells in the SV11 natural repertoire has precluded direct observation of the effect of endogenous Tag expression on higher avidity T cells specific for this epitope in tumor-bearing mice. Therefore, the purpose of the present investigation was to examine the potential for high-avidity T-CD8 cells specific for the immunorecessive Tag epitope V to recognize endogenous Tag in vivo; the fate and behavior of Tag epitope V-specific T cells in the tumor-antigen host environment; and the ability of Tag epitope V-specific T cells to control tumor progression upon adoptive transfer into SV11 mice bearing advanced-stage SV40 Tag-expressing choroid plexus tumors.

In order to study the behavior of naive Tag-V specific T-CD8 cells in response to Tag-expressing tumors, epitope V-specific T-CD8 cells from TCR transgenic mice (TCR-V cells) were transferred into SV11 mice with advanced tumors. Line TCR-V transgenic mice express the TCR α and β chains derived from the epitope V-specific clone Y-5 (Tanaka et al., 1989) on the C57BL/6 background and have been previously characterized (Otahal et al., 2005; Schell, 2004). The epitope V-specific Y-5 clone was generated in a C57BL/6 mouse immunized with a Tag variant cell line expressing only the immunorecessive epitope V (Tanaka et al., 1989). This clone is thus representative of a Tag-V specific clone from a normal T cell repertoire. TCR-V mice were bred with B6.SJL mice so that donor CD45.1+ TCR-V cells could be distinguished from CD45.2+ host cells in the SV11 mice. Thus, TCR-V cells served as naïve donor lymphocytes, allowing the response of Tag epitope V-specific T-CD8 cells to be monitored following adoptive transfer into tumor-bearing SV11 mice.
RESULTS

Naïve TCR transgenic T-CD8 cells (TCR-V) recognize endogenous epitope V in SV11 mice

Naïve TCR-V lymphocytes (Figure 13A) were adoptively transferred into 85-day old SV11 mice bearing advanced choroid plexus tumors or into Tag transgene-negative littermates, hereafter referred to as B6 mice (Figure 16). Some of the mice were immunized at the time of transfer with Tag transformed B6 cells expressing a Tag variant in which the immunodominant epitopes I, II/III, and IV were inactivated (B6/V-only Tag) (Figure 13B). Seven days post transfer, splenocytes were analyzed for frequency and activation status of recovered TCR-V cells, detected by staining with MHC class I tetramers. Immunized B6 mice demonstrated a 20-fold increase in total tetramer-V+ T cells over the baseline detected in unimmunized B6 mice – 2x10^6 cells and 1x10^5 cells - respectively (Figure 17A). Immunization of B6 mice induced an activated phenotype in TCR-V T-CD8 cells consistent with their extensive accumulation. Nearly the entire population expressed high levels of CD44 while 75% down-regulated the LN homing receptor L-selectin. In naïve SV11 mice, the number of TCR-V cells increased 4-fold above that detected in naïve B6 mice, representing 4x10^5 cells in the spleen. Consistent with this modest expansion, recovered TCR-V cells had up-regulated expression of CD44, indicating recognition of the endogenous Tag. However, only a small population of cells – 19% - downregulated L-selectin. TCR-V T cells expanded to 8x10^5 splenocytes in SV11 mice following immunization, an 8-fold increase above that detected in naïve B6 mice, but only a 2-fold increase over the number in naïve SV11 mice. Approximately 50% of these tetramer-V+ T-CD8 cells had lost expression of L-selectin following immunization, consistent with the two-fold increase in TCR-V T cell frequency. All tetramer-V+ T-CD8 cells detected were of donor origin as indicated by the homogeneous expression of CD45.1. These results indicate that naïve TCR-V T cells recognize the endogenous Tag in tumor-bearing SV11 mice, but only modestly accumulate above input levels. Subsequent immunization further expanded this population, although the levels never reached that achieved in immunized B6 hosts (Figure 17A).

Given that TCR-V cell accumulation in the spleens of SV11 mice was limited following recognition of endogenous Tag, their proliferative potential was assessed in vivo. TCR-V cells were labeled with CFSE prior to adoptive transfer into 85-day old SV11 mice or B6 littermates (Appendix A). The CLN is believed to be the reservoir for antigens that drain from the brain.
FIGURE 16

General Adoptive Immunotherapy Protocol

The following general protocol was used for adoptive immunotherapy of SV11 mice and control transgene-negative littermates (B6 mice). Variations on the general protocol are described for particular experiments as indicated in the data section.

**TCR-V Cell Adoptive Transfer:** Erythrocyte-depleted lymphocytes derived from spleens and peripheral lymph nodes of TCR-V/CD45.1 transgenic mice were injected intravenously in 0.4 mL into the tail veins of 85 day-old SV11 or B6 transgene-negative littermates at a dose of $5 \times 10^6$ clonotypic TCR-V cells/mouse.

**Immunization:** B6/V-only Tag cells were injected in 0.5 mL via the intraperitoneal route at a dose of $5 \times 10^7$ B6/V-only Tag cells/mouse. Primary immunizations were administered on the day of adoptive transfer; booster immunizations were administered on day 7 post adoptive transfer.

**Ex Vivo Analysis:** Spleens, cervical lymph nodes, and brains were harvested and assessed as described per experiment.
FIGURE 16

General Protocol for TCR-V Cell Adoptive Transfer and Immunization of SV11 Mice

ADOPTIVE TRANSFER
intravenous naive TCR-V cells

85 days of age

EX VIVO ANALYSIS
Isolate TCR-V cells from spleen, CLN, and brain

T antigen epitope-V specific IMMUNIZATION
intraperitoneal injection with Tag epitope-V only cells

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A. Naïve TCR-V lymphocytes (5x10^6 cells/mouse) were adoptively transferred into 85 day old SV11 mice or Tag transgene-negative (B6) littermates. Some mice were immunized (prime) at the time of transfer with B6/V-only Tag cells (5x10^7 cells). Seven days post transfer, splenocytes were analyzed for frequency of CD8+/tetramer-V+ cells and indicated as percent of total CD8+ cells and total number of epitope-specific splenocytes (left panels). Total numbers represent the mean of 3 mice/group and the experiment was performed 3 times. Recovered cells were co-stained for donor origin (CD45.1), and activation status (CD44 and L-selectin). The percent of CD45.1+, CD44hi and L-selectinlo cells is indicated.

B. TCR-V cells were labeled with CFSE prior to adoptive transfer of 5x10^6 cells into 85 day old SV11 mice or B6 littermates. At 7 days post transfer, CD8+/Tet-V+ cells from the spleen and CLN were examined for proliferative status. The percent of CD8+/Tet-V+ cells that remain undivided (right marker), had divided 1-6 times (center marker), or greater than 6 times (left marker) is indicated.
FIGURE 17

Naïve TCR-V T-CD8 Cells Recognize Endogenous Wild Type Tag in SV11 Mice

A

B6
TCR-V only 1.8% (1x10^5)

B6
TCR-V + prime 16% (2x10^4)

SV11
TCR-V only 2.7% (4x10^4)

SV11
TCR-V + prime 5.6% (8x10^5)

Tetramer-V

Gated on Tet-V+CD8+

cells

97% 12% 3%

99% 98% 75%

97% 97% 19%

98% 98% 50%

CD45.1
CD44
L-selectin

CFSE

B

Spleen

CLN

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The Journal of Immunology 2006, 177: p.258)
(Cserr and Knopf, 1992). Therefore, at 7 days post TCR-V cell transfer, lymphocytes from the spleen and CLN were examined for the proliferative status of donor epitope-V specific T-CD8 cells (Figure 17B). Immunization of B6 mice resulted in loss of CFSE label among most TCR-V cells recovered from the spleens and a prominent population in the CLN, indicating multiple rounds of division. Upon transfer to SV11 mice, 40% of TCR-V cells in the spleen underwent 7 or more divisions, while 28% appeared to be in intermediate rounds of division (between 2-4 cycles). Subsequent immunization of SV11 mice yielded a further 2-fold increase in the percentage of cells in the spleen that had lost the CFSE label (Figure 17B). TCR-V cells were also detected in the draining CLN of naïve SV11 mice. Unlike in the spleen, immunization only minimally increased the percentage of TCR-V cells that had undergone 7 or more divisions in the CLN. These results indicate that recognition of endogenous Tag in naïve SV11 mice leads to a modest degree of expansion consistent with the presence of proliferating cells. Immunization doubled the overall expansion of TCR-V cells detectable in the spleen and lymphoid organs of SV11 mice. However, while the proportion of dividing cells in immunized B6 and SV11 mice is the same, fewer total TCR-V cells accumulate in the spleens of SV11 mice.

Differential kinetics of TCR-V T cell activation and accumulation in response to endogenous Tag versus immunization

There are several potential explanations for the failure of TCR-V cells to accumulate in the spleens and CLN of naïve SV11 mice by 7 days post adoptive transfer. These include: i) an earlier peak in expansion; ii) migration to a different anatomic site following activation; or iii) deletion. In order to examine whether the peak of the proliferative response preceded our day 7 analysis time point, a kinetic study was performed to monitor the frequency, activation status, and proliferation of TCR-V cells in the spleen, draining CLN, and non-draining inguinal lymph nodes (ILN) at specified time points following TCR-V cell transfer. The frequency of tetramer-

V+/CD8+ T cells recovered from the spleen, CLN, and ILN was assessed at 24, 48, and 96 hours as well as 7 and 14 days post TCR-V cell transfer.

In immunized B6 mice, the peak of the response in the spleen occurred on day 7, with TCR-V cells comprising $1.8 \times 10^6$ cells per spleen (Figure 18A). By contrast, TCR-V cell frequency in the spleen of naïve SV11 mice peaked at day 4, comprising an average of $5 \times 10^5$ cells per spleen. Although the frequency of TCR-V T cells in the spleen at day 4 post-adoptive
A and B. Naïve TCR-V cells were transferred into groups of SV11 or B6 hosts (3 mice/group). Some mice in each group also received B6/V-only T Ag immunization. A. The total number of CD8+/tetramer-V+ cells recovered from the spleen, CLN, and ILN was assessed at 24, 48, and 96 hours as well as 7 and 14 days post transfer.

B. The expression of activation markers CD69, and CD44 was assessed on gated populations of CD8+/Tet-V+/CD45.1+ cells recovered from the spleen and CLN at 2, 6, 24, 48, 72, and 96 hours post transfer of TCR-V cells.

C. Naïve TCR-V cells were CFSE-labeled prior to adoptive transfer into groups of 85 day old SV11 or B6 mice (4 mice/group). Some SV11 or B6 mice also received immunization with B6/V-only Tag cells on the day of transfer. Proliferation of CD8+/Tet-V+/CD45.1+ recovered cells was assessed in the spleen and CLN at 2 days and 4 days post transfer.
FIGURE 18

A

SPLEEN

# TCR-V cells (x10^4)

CLN

# TCR-V cells (x10^3)

days post-adoptive transfer

days post-adoptive transfer

B

CD69

% TCR-V cells

CD95 hi

0 2 4 6 8 10 20 40 60 80

hours post-adoptive transfer

CD44

% TCR-V cells

CD44 hi

0 20 40 60 80 100

CLN

C

DAY 2

DAY 4

SPLEEN

CLN

SPLEEN

CLN

B6

TCR-V only

B6

TCR-V + prime

SV11

TCR-V only

SV11

TCR-V + prime

CFSE
transfer was similar in both naïve and immunized SV11 mice, immunization delayed the peak of TCR-V T cell accumulation until day 7. The kinetics of TCR-V cell accumulation in the CLN of B6 mice was similar to that observed in the spleen, peaking 7 days post immunization (Figure 18A). In contrast, the frequency of TCR-V cells increased dramatically in the CLN within the first 48 hours post adoptive transfer into both naïve and immunized SV11 mice. The magnitude of the response in B6 mice never reached the peak levels observed in the CLN of SV11 mice. This rapid accumulation of TCR-V cells in the CLN of SV11 mice is likely due to the presence of endogenous Tag draining from the choroid plexus. The kinetics of T cell expansion and contraction in the CLN of SV11 mice was similar regardless of whether the mice were immunized. As a control for the tumor-draining CLN, a third anatomic site - the non-draining ILN - was also analyzed. The kinetics of TCR-V cell accumulation in the ILN paralleled the results measured in the spleen (data not shown). These results reveal that 1.) the kinetics of TCR-V accumulation in the spleen is similar in SV11 and B6 mice following immunization, despite less extensive expansion in SV11 mice, 2.) immunization of SV11 mice delays the peak of TCR-V accumulation in the spleen and 3.) TCR-V cells accumulate rapidly in the CLN in response to the endogenous Tag.

Endogenous Tag triggers rapid activation of naïve TCR-V cells in SV11 mice

The accelerated accumulation of TCR-V cells in the tumor-draining CLN of SV11 mice suggests that TCR-V cells encounter endogenous Tag earlier than Tag derived from cellular immunization. To address this question, the initial response of TCR-V cells to endogenous Tag in naïve SV11 mice was compared with the response to immunization in B6 mice. The expression of activation markers CD69 and CD44 was assessed on gated populations of CD8+ Tet-V+CD45.1+ cells at 2, 6, 24, 48, 72, and 96 hours post transfer of TCR-V cells. CD69 was initially detected on TCR-V T cells recovered from the spleens of B6 mice between 24 and 48 hours after adoptive transfer and immunization (Figure 18B, left panels). In the CLN, up-regulation of CD69 was not observed until 72 hours. A more rapid kinetic profile was observed in naïve SV11 mice. Expression of CD69 on TCR-V T cells was first detected at 6 hours on cells from the spleen and 2 hours on cells from the CLN. In addition, the percentage of TCR-V T cells that up-regulated CD69 after transfer into SV11 mice was significantly higher than in immunized B6 mice. This result might be explained by a larger proportion of TCR-V T cells being initially activated by the endogenous Tag than by immunization. Alternatively, since CD69
expression is transient, TCR-V T cells activated by immunization might more rapidly down-regulate CD69 expression than T cells activated by the endogenous Tag. Thus, expression of CD44, an activation marker that retains stable high-level expression on the surface of antigen experienced cells, was also examined. Prior to adoptive transfer, approximately 12% of TCR-V cells were CD44\textsuperscript{hi} and upon transfer to naïve B6 mice, 20-30% of TCR-V cells recovered from the spleen and CLN were CD44\textsuperscript{hi}. This increase is not likely due to homeostatic proliferation of transferred TCR-V cells since they fail to proliferate in B6 hosts (Figure 17B), but might be due to preferential retention of CD44\textsuperscript{hi} cells in the lymphoid organs of naïve mice. Following immunization of B6 mice, 75% and 50% of TCR-V cells in the spleen and CLN, respectively, were CD44\textsuperscript{hi} by 72 hours. By 96 hours post immunization, 90% of the TCR-V cells in both organs were CD44\textsuperscript{hi}. In contrast, upon transfer to naïve SV11 mice, 75% of the TCR-V cells in the spleen were CD44\textsuperscript{hi} by 48 hours, 24 hours earlier than what was observed in immunized B6 mice. A similar profile was observed in the CLN, where 80% of TCR-V cells were CD44\textsuperscript{hi} by 48 hours. Thus, the kinetics of CD44 expression suggest that TCR-V T cells are activated rapidly against the endogenous Tag in SV11 mice with the effects of exogenous immunization delayed for 24-48 hours.

Given that TCR-V T cells are phenotypically activated very early post-transfer into SV11 mice (Figure 18B), it was plausible that they also undergo an earlier proliferative burst in the tumor-bearing environment. To address this question, proliferation of CFSE-labeled TCR-V cells was assessed at 2 and 4 days following adoptive transfer. TCR-V cells transferred to unimmunized B6 mice remained undivided at both time points (Figure 18C). Proliferating TCR-V T cells could be detected in immunized B6 mice by day 4, but not day 2, following immunization. In contrast, TCR-V cells transferred to naïve SV11 mice were actively dividing by day 2 in both the spleen and CLN, although a larger proportion of cells in the CLN had divided. The effects of immunization of SV11 mice were not realized on day 2, but by day 4 immunized SV11 mice had an increased proportion of cells that had divided in both the spleen and CLN compared to naïve SV11 mice. This result is consistent with the accumulation of higher numbers of TCR-V T cells in the lymphoid organs of immunized versus naïve SV11 mice.

Taken together, these results indicate that the earlier peak in TCR-V T cell accumulation in naïve SV11 mice versus immunized B6 mice is due to the 24-48 hour head start in TCR-V T cell activation and proliferation. The finding that subsequent immunization of SV11 mice confers similar kinetics of T cell expansion and contraction as found in B6 mice might be
explained by increased triggering of naïve T cells or by enhanced survival of activated cells (Masopust and Ahmed, 2004). However, despite comparable kinetics, TCR-V T cell frequency in immunized SV11 mice never reached the levels observed in B6 mice, suggesting that cells exposed to endogenous Tag in SV11 mice are less responsive to immunization administered on the day of adoptive transfer.

**Naïve TCR-V cells acquire effector function in response to endogenous Tag in SV11 mice**

To determine whether TCR-V T cells responding to the endogenous Tag acquire effector function in tumor-bearing SV11 mice, both IFN-γ production (Appendix B) by donor TCR-V T cells and in vivo killing (Appendix C) of epitope V-pulsed target cells was assessed in tumor-bearing mice. Naïve TCR-V cells were transferred into SV11 or B6 mice at 85 days of age. Some mice were immunized with B6/V-only Tag cells on the day of adoptive transfer. Seven days later, spleens were harvested and the frequency of peptide-specific IFN-γ-producing cells was determined. The number of CD8+ splenocytes producing IFN-γ in response to epitope V peptide was directly compared to the number of tetramer-V+ CD8+ splenocytes to estimate the proportion of TCR-V cells harboring effector function (Figure 19A). In immunized B6 mice, 2.1x10^6 cells were tetramer-V+ and 1.6x10^6 cells secreted IFN-γ, indicating that the majority of tetramer-V+ cells were functional. In naïve B6 mice, the frequency of CD8+ cells that produced IFN-γ in response to Tag-V peptide was at background levels (equivalent to control peptide). In naïve SV11 mice, 4x10^5 splenocytes were tetramer-V+ and approximately half this number of cells produced IFN-γ, demonstrating that TCR-V T cells acquired effector function following activation in naïve SV11 mice. Upon immunization of SV11 mice, the number of tetramer-V+ cells doubled to 8x10^5 cells and approximately 75% of this number produced IFN-γ in response to Tag epitope-V. These data suggest that upon immunization, the fraction of TCR-V cells with effector function is similar to that achieved in B6 mice, although the magnitude of the response is not as high.

Assays were also performed to determine the in vivo cytotoxic activity of TCR-V cells (Figure 19B). TCR-V cells were transferred to SV11 or B6 mice with or without immunization. Seven days later, equal numbers of differentially-labeled peptide-V and control peptide (Flu-NP) pulsed target cells were injected intravenously and assessed for elimination in the spleen after 12 hours. Upon transfer into unimmunized B6 mice, no specific loss of either population was
Naïve TCR-V Cells Acquire Effector Function in Response to Endogenous Tag in SV11 Mice.

A. Naïve TCR-V cells were transferred into groups of SV11 or B6 mice at 85 days of age (3 mice/group). Some mice were immunized with B6/V-only Tag cells on the day of adoptive transfer. Seven days later, spleens were harvested and the total number of epitope V specific CD8+ cells was determined by staining for intracellular interferon-g production or tetramer analysis.

B. TCR-V cells were transferred to SV11 or B6 mice with or without immunization. Seven days later, equal numbers of differentially-labeled peptide-V (5 μM CFSE) and control peptide-Flu (0.5 μM CFSE) pulsed target cells were injected intravenously and assessed for elimination in the spleen after 12 hours. The percent specific cytotoxicity of epitope V-pulsed targets is indicated as compared to B6 mice that received neither TCR-V T cells nor immunization (target population).
FIGURE 19

Naïve TCR-V Cells Acquire Effector Function in Response to Endogenous Tag in SV11 Mice.

A

B

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detected (Figure 19B). In immunized B6 mice, virtually all peptide-V pulsed targets were eliminated, indicating Tag-V specific function by TCR-V cells in vivo. In unimmunized SV11 mice, 47% of epitope-V pulsed targets were eliminated, and this percentage was further increased to 68% in immunized SV11 mice. Target cells were not eliminated from SV11 mice that did not receive TCR-V T cells (data not shown). Thus, TCR-V cells are capable of developing effector function in response to endogenous Tag in tumor-bearing SV11 mice, even in the absence of immunization.

**Tag epitope V-targeted immunization promotes TCR-V cell entry into the brain**

The discrepancy in the total number of TCR-V T cells that accumulate in the spleens of SV11 versus B6 mice following immunization might be explained by deletion of T cells responding to endogenous tumor antigen (Kurts et al., 1997; Lyman et al., 2005; Robbins et al., 2004) or alternatively, their migration to the tumor site (Kedl and Mescher, 1997; Yu et al., 2004; Yu et al., 2005). In order to assess the potential for naïve TCR-V cells to infiltrate choroid plexus tumors, TCR-V cells were transferred into SV11 or B6 mice with and without immunization and their presence in spleens and brains was assessed 7 days later. Frequencies of TCR-V cells in the spleen represented 3.8% and 2.3% of CD8+ cells in naive SV11 and B6 mice, respectively (Figure 20). No TCR-V cells infiltrated the brains of either SV11 or B6 mice without immunization, indicating that priming of TCR-V T cells against the endogenous Tag fails to result in significant accumulation of T cells in the brains of tumor-bearing SV11 mice.

Upon primary immunization, TCR-V cell frequency increased to 6.4% and 22% of CD8+ splenocytes in SV11 and B6 mice, respectively. In addition, immunization resulted in a significant influx of TCR-V T cells into the brains of both groups, representing 5.5% and 4.7% of CD8+ T cells in SV11 and B6 mice, respectively (Figure 20). The presence of TCR-V cells in the brains of both strains of mice at this time was most likely the result of the acute response to immunization, as similar proportions of T cells reactive toward the immunodominant Tag epitope IV were previously observed to enter the brains of B6 mice following immunization (Schell and Tevethia, 2001). In the present study, we noted that similar numbers of TCR-V T cells infiltrated the brains of SV11 and B6 mice despite 3-fold lower numbers of TCR-V T cells in the spleens of SV11 mice, suggesting some preferential accumulation in SV11 brains. Thus, specific immunization, but not endogenous Tag, promoted TCR-V cell access to the brain regardless of
FIGURE 20

Immunization Promotes TCR-V Cell Entry into the Brains of Both SV11 and B6 Mice

TCR-V cells were transferred into groups of SV11 or B6 mice with and without immunization. The frequency of CD8+/Tet-V+ cells recovered from the spleen and brain was assessed 7 days later by MHC tetramer staining.

Data shown is the mean of 3 separate experiments, which included 3 mice/group. Error bars indicate standard deviation from the mean.
FIGURE 20

Immunization Promotes TCR-V Cell Entry into the Brains of Both SV11 and B6 Mice

(REPRODUCED FROM RYAN AND SCHELL
THE JOURNAL OF IMMUNOLOGY 2006, 177: P.261)
whether Tag was expressed in the choroid plexus or tumor was present.

**Adoptively transferred TCR-V cells accumulate in SV11 tumors following secondary immunization and express high levels of CD38**

Previous work demonstrated that the residual epitope V specific T-CD8 cells in SV11 mice are optimally expanded following a prime and boost regimen (Schell, 2004). Given that primary immunization promoted TCR-V cell expansion in the spleen and promoted migration to the brain (Figure 20), the persistence of TCR-V cells at later time points was also assessed, and the potential for further accumulation following a booster immunization was examined. TCR-V cells were transferred into SV11 or B6 mice +/- primary immunization. Seven days later, spleens and brains from representative mice were analyzed for the presence of CD8+ Tet-V+ cells, while some of the primed mice from each group were boosted with B6/V-only Tag immunization. All remaining mice were analyzed 20 days following the initial TCR-V cell transfer, at 105 days of age (Figure 21). The frequencies of TCR-V cells detected at day 7 following adoptive transfer +/- immunization were similar to the data presented in Figure 20 (Table 1). By 20 days post transfer, TCR-V cells were undetectable in the spleens and brains of both naïve SV11 and B6 mice. In mice that received primary immunization, 1.2% of SV11 CD8+ splenocytes and 10% of B6 CD8+ splenocytes were Tet-V+ at 20 days post-transfer. This represents a 4-fold and 2-fold respective decrease in the cell frequencies observed in SV11 and B6 mice at day 7. In the brains of primed B6 mice, 3% of CD8+ cells were Tet-V+, whereas as 20% of CD8+ cells were Tet-V+ in SV11 brains. These values represent a 2-fold decrease for B6 mice but a 3-fold increase for SV11 mice. Thus, TCR-V T cells continue to accumulate at the tumor site between days 7 and 20 following primary immunization.

The effect of boosting on expansion of TCR-V T cells in SV11 mice (Table 1) was subsequently assessed. In the spleens of primed/boosted B6 mice, TCR-V cells expanded to 25% of CD8+ cells, a 1.3-fold increase over the level detected 7 days after the primary immunization. In the brains of these same mice, 7% of CD8+ cells were specific for epitope V, representing only a 1.1-fold increase in the level detected at day 7. The frequency of CD8+ T cells that were Tet-V+ in SV11 mice following the prime/boost regimen was 5% and 76% in the spleen and brain, respectively. This represented a 1.6-fold decrease in the spleen, but a 10.5-fold increase at the tumor site in the brain. This dramatic increase in TCR-V accumulation was
Protocol for TCR-V Cell Adoptive Transfer Followed by Prime and Boost Immunization

TCR-V cells were transferred into 85 day old SV11 or B6 mice. Some mice were administered primary immunization with $5 \times 10^7$ B6/V-only Tag cells on the day of adoptive transfer. Some of the mice that had received primary immunization were also administered a secondary immunization with $5 \times 10^7$ B6/V-only Tag cells 7 days after adoptive transfer and prime (92 days of age). Lymphocytes were isolated and analyzed from spleens and brains of all mice on day 20 following TCR-V cell transfer, at 105 days of age.
FIGURE 21

Protocol for TCR-V Cell Adoptive Transfer + Tag-V Targeted Prime/Boost Immunization

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TCR-V cells</th>
<th>primary immunization</th>
<th>booster immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>A transfer only</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B transfer + prime</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C transfer + prime/boost</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

85 days old | 92 days old | 105 days old
TCR-V cells + prime | boost | analysis
TCR-V cells were transferred into 85 day old SV11 or B6 mice +/- primary immunization with B6/V-only Tag cells. After 7 days, lymphocytes recovered from spleens and brains of representative mice were analyzed for the presence of CD8⁺/Tet-V⁺ cells, while half of the remaining primed mice from each group were boosted with V-only Tag immunization. All remaining mice were analyzed on day 20 following TCR-V cell transfer, at 103 days of age. TCR-V cell frequencies in the spleen and brain are represented as percent of CD8⁺ cells and percent of total cells. Data shown is the mean of 3 separate experiments, which included 3 mice/group.
Table 1.

Accumulation of TCR-V Cells in the Brains of SV11 Mice Following Secondary Immunization

<table>
<thead>
<tr>
<th>Strain</th>
<th>Prime</th>
<th>Boost</th>
<th>Analysis</th>
<th>Tet-V⁺ Cells</th>
<th>% of total cells</th>
<th>% of CD8⁺ cells</th>
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<td></td>
<td></td>
<td></td>
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<td>Brain</td>
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<td>0.9</td>
<td>19.0</td>
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<td>day 20</td>
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</tr>
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<td></td>
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<td>none</td>
<td>day 7</td>
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<td>0.8</td>
<td>22.0</td>
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</tr>
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</table>

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also reflected in that TCR-V T cells represented 22% of total brain infiltrating lymphocytes in SV11 mice that received both a prime and boost. These data indicate that TCR-V T cells preferentially accumulate at the tumor site in SV11 mice.

The dramatic difference in TCR-V cell expansion and accumulation is depicted in Figure 22A. In the absence of immunization, TCR-V cells did not expand above 3% of total CD8+ cells in the spleen and did not infiltrate the brain when analyzed 7 days post-transfer. When SV11 mice were immunized on the day of adoptive transfer and assessed 7 days later, the frequency of TCR-V cells moderately increased in the spleen (5.6% of CD8+ cells) and a barely detectable population was observed in the brain (2% of CD8+ cells). However, when SV11 mice were administered both a primary and booster immunization on the day of transfer and 7 days later, respectively, and assessed on day 20 post-transfer, TCR-V cells had accumulated significantly in SV11 brains (76% of CD8+ cells), while a modest proportion remained in the periphery (4% of CD8+ cells).

Since brain infiltration was only observed following immunization, it was possible that activation of TCR-V cells with V-only specific immunization resulted in a licensing signal allowing TCR-V cells access to the brain. Several different markers have been reported to influence homing to the brain, including CD38, a T cell marker that binds to PE-CAM expressed on endothelium (Fabry et al., 1992; Qing et al., 2001). For SV11 mice that had been adoptively transferred with TCR-V cells and primed and boosted, splenocytes and brain lymphocytes were assessed for TCR-V expression of CD38 (Figure 22B). A fraction of the TCR-V cell population in the spleen had up-regulated CD38 (green), while a homogeneous population in the brain all expressed the marker at high levels (red), suggesting that CD38 contributes to the ability for TCR-V cells to gain access to the brain. A fraction of TCR-V cells in B6 spleens expressed levels of CD38 similar to the levels expressed by TCR-V splenocytes in SV11 mice (yellow). The remaining non-Tag specific CD8+ cells in SV11 spleens demonstrated low-level expression of CD38 (blue). While these data are not conclusive for the ability of immunization to induce expression of CD38, these results suggest that PE-CAM/CD38 mediated adhesion to cerebral vasculature may participate in extravasation of activated T-CD8 from the periphery into the brain.
TCR-V cells were transferred into 85 day old SV11 or B6 mice and given (i.) no immunization; (ii.) primary immunization on the day of transfer; or (iii.) both primary (day 0) and booster (day 7) immunizations. Lymphocytes isolated from spleens and brains were analyzed on day 20 following TCR-V cell transfer, at 105 days of age.

A. Recovered CD8+/TCR-V+ cells were co-stained for CD8 and Tetramer-V.

B. For SV11 or B6 mice that had been adoptively transferred with TCR-V cells and primed and boosted, splenocytes and brain lymphocytes were assessed for TCR-V expression of CD38.
FIGURE 22

Adoptively Transferred TCR-V Cells Accumulate in SV11 Tumors Following Secondary Immunization and Express High Levels of CD38

A.

<table>
<thead>
<tr>
<th>SPLEEN</th>
<th>BRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR-V cells only</td>
<td>3%</td>
</tr>
<tr>
<td>TCR-V cells + primary immunization</td>
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</tr>
<tr>
<td>TCR-V cells + prime/boost immunization</td>
<td>4%</td>
</tr>
</tbody>
</table>

B.

red: Tet-V+ SV11 brain  
green: Tet-V+ SV11 spleen  
blue: Tet-V- SV11 spleen  
yellow: Tet-V+ B6 spleen  

CD38
Brain-infiltrating TCR-V cells detected in SV11 mice are activated and functional

Several reports in both mouse and human studies have indicated that CD8+ T cells recovered from tumors are anergic (Blohm et al., 2002; Lee et al., 1999b). Thus, the activation state and functional potential of TCR-V cells recovered from SV11 mice that had been primed and boosted was assessed. In the spleens of both B6 and SV11 mice, a homogenous population expressed CD44, indicating that all cells were antigen experienced (Figure 23A). Additionally, in both of these groups the majority of cells had down-regulated L-selectin (L-selectin\textsuperscript{lo}), although splenocytes from SV11 mice also contained a population of L-selectin\textsuperscript{hi} cells. TCR-V cells recovered from the brains of both B6 and SV11 mice were homogeneously CD44\textsuperscript{hi} and L-selectin\textsuperscript{lo}, indicating that only highly activated cells were present in the brain.

To determine whether these phenotypically activated cells retained effector cytokine function, brain-infiltrating TCR-V cells in 103 day old B6 and SV11 mice that had received adoptive transfer/prime/boost were assessed for ex vivo production of IFN\textgreek{y} in response to Tag-V peptide stimulation. The percentage of Tet-V+ cells detected in the spleens of B6 and SV11 mice at this time was 25% and 5% of CD8+ cells, respectively (Figure 23B). A proportion of these cells made IFN\textgreek{y} - with 15% and 1.8% producing Tag-V peptide stimulated cytokine in B6 and SV11 mice, respectively. In the brain, 13% of CD8+ cells in B6 mice and 76% of CD8+ cells in SV11 mice were tetramer-V+. A population of brain-derived lymphocytes from both B6 (4% of CD8+ cells) and SV11 (27% of CD8+ cells) mice also produced IFN\textgreek{y} in response to epitope V peptide (Figure 23B), indicating that at least a subset of TCR-V cells from the tumor site of SV11 mice maintained the capacity to secrete cytokine.

An alternative mechanism for control of tumor progression by CD8+ T cells is the production of other cytokines, such as TNF\textgreek{a}. Thus, the capacity for TCR-V cells isolated from B6 and SV11 mice at 105 days of age to secrete IFN\textgreek{y} versus TNF\textgreek{a} following priming and boosting was directly compared. A significant proportion of SV11 splenocytes (3% of CD8+ cells) and brain lymphocytes (28% of CD8+ cells) produced IFN\textgreek{y} in response to Tag-V peptide stimulation and assayed via intracellular cytokine stain (Figure 24A). Additionally, in the same assay, a proportion of TCR-V cells also made TNF\textgreek{a} - with 8% of total CD8+ splenocytes and 15% total CD8+ brain lymphocytes producing this cytokine (Figure 24B). These data demonstrate that multiple functional mechanisms are induced in TCR-V cells that accumulate in
TCR-V cells were transferred into 85 day old SV11 or B6 mice and given both primary (day 0) and booster (day 7) immunizations. Lymphocytes isolated from spleens and brains were analyzed on day 20 following TCR-V cell transfer, at 103 days of age.

A. Recovered CD8+/TCR-V+ cells were co-stained for CD44 and L-selectin. The percent of CD44<sup>hi</sup> and L-selectin<sup>lo</sup> cells is indicated.

B. The frequency of peptide V-specific IFN-γ-producing CD8+ cells in the spleen and brain was determined via intracellular cytokine stain, and compared to the frequency of CD8+ cells that stained positive for tetramer-V. Values indicate the percentage of CD8+ cells specific for epitope V and have been corrected by subtraction of background values obtained using control tetramer or following stimulation with control peptide.
FIGURE 23

Brain-infiltrating TCR-V Cells Detected in SV11 Mice Are Activated and Functional

(A) (Gated on CD8+Tet-V+ Cells)

B6

SV11

CD44

L-selectin

CD44

L-selectin

SPLEEN

BRAIN

25%

5%

13%

76%

15%

1.8%

4%

27%

(B) (Reproduced from Ryan and Schell
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TCR-V cells were transferred into 85 day old SV11 or B6 mice and given both primary (day 0) and booster (day 7) immunizations. Lymphocytes isolated from spleens and brains were analyzed on day 20 following TCR-V cell transfer, at 105 days of age. The frequency of peptide V-specific IFN-γ-producing CD8+ cells in the spleen and brain was determined via intracellular cytokine stain (A.), and compared to the frequency of peptide V-specific TNF-α-producing CD8+ cells in the spleen and brain as determined via intracellular cytokine stain (B.). Values indicate the percentage of CD8+ cells specific for epitope V and have been corrected by subtraction of background values obtained following stimulation with control peptide. Error bars indicate standard deviation from the mean.
FIGURE 24

A. IFNγ production post adoptive transfer + prime/boost

B. TNFα production post adoptive transfer + prime/boost
SV11 tumors following immunotherapy.

**TCR-V cells infiltrate choroid plexus tumor stroma following secondary immunization**

To determine whether TCR-V cells detected among brain infiltrating lymphocytes could migrate into the tumor stroma in SV11 mice, immunohistochemical analyses were performed. Frozen sections were prepared from SV11 and B6 brains following (i) no treatment, (ii) TCR-V cell transfer + primary immunization, and (iii) TCR-V cell transfer + prime/boost. Brains were harvested at day 20 following TCR-V cell adoptive transfer and sections stained for CD8. In SV11 mice receiving no treatment, brain sections were devoid of CD8 staining (Figure 25A). CD8+ cells were found within the tumor tissue of SV11 mice that received primary immunization (Figure 25B), but the density of CD8+ cell infiltration increased following the booster immunization (Figure 25C). Cells staining positive for CD8 were localized only to the tumor stroma and were not detected throughout the brain parenchyma in SV11 mice. Staining was not detected in the absence of primary anti-CD8 antibody (data not shown). While B6 mice lack tumor mass in the choroid plexus, some CD8+ cells were detected scattered throughout the brain parenchyma in mice that had received TCR-V cell transfer/prime/boost (Figure 25D), but these cells did not localize to the choroid plexus. Thus, histological analysis of SV11 brains supports the quantitative flow cytometric analysis of brain-infiltrating CD8+ cells in SV11 and B6 mice, indicating that CD8+ cells specifically infiltrate the SV11 tumor stroma following TCR-V cell transfer combined with prime/boost.

In order to confirm that brain-infiltrating CD8+ cells were actually TCR-V cells, donor lymphocytes from TCR-V transgenic mice that had been crossed with GFP+ mice (such that all donor TCR-V cells were also GFP+) were adoptively transferred into SV11 mice. Following the same immunotherapeutic regimen of TCR-V cell transfer/prime/boost, spleens and brains were harvested from SV11 and B6 mice 20 days post TCR-V cell transfer. Upon microscopic examination, GFP+ cells were abundant in SV11 brain sections and similar to the distribution of CD8+ cells in the immunohistochemical analysis, these cells appeared to cluster at specific zones in the tumor tissue (Figure 25E). Co-staining of SV11 sections with anti-CD8 revealed the specific accumulation of GFP+CD8+ cells within the tumor and the virtual absence of these cells from the rest of the brain parenchyma visualized with DAPI (Figure 25F). Some GFP+
FIGURE 25

TCR-V Cells Infiltrate SV11 Choroid Plexus Tumors Following Secondary Immunization

Panels A-D. SV11 and B6 mice were divided into 3 treatment groups: (i) no treatment, (ii) TCR-V cell transfer + primary immunization at 85 days of age and (iii) TCR-V cell transfer + primary immunization at 85 days of age + booster immunization 7 days later. Brains were harvested at day 20 following TCR-V cell adoptive transfer (103 days old) and frozen sections were prepared and stained for CD8 (brown). Representative sections are shown for SV11 mice that received no treatment (A); TCR-V + prime (B); TCR-V + prime + boost (C) and B6 mice that received TCR-V + prime + boost (D). All images are shown at 200x magnification. Panels E and F. 85 day old SV11 and B6 mice were adoptively transferred with 5x10^6 clonotypic donor lymphocytes from GFP+/TCR-V mice. Mice were primed on the day of adoptive transfer (day 0) and boosted 7 days later. Spleens and brains were harvested 7 days following booster immunization (103 days of age). E. Frozen sections were prepared and stained with DAPI (blue) to visualize the tumor. GFP+/TCR-V cells are visualized as green cells infiltrating the tumor (100x magnification). F. Some specimens were co-stained for CD8 (red) (200x magnification). Abbreviations: T, tumor; P, brain parenchyma; V, ventricle.

G. Brains were also processed and assessed flow cytometrically for the presence of CD8+/Tet-V+ cells (right panel) that also express GFP (left panel).
FIGURE 25

(A) SV11 no treatment
(B) SV11 TCR-V + prime
(C) SV11 TCR-V + prime/boost
(D) B6 TCR-V + prime/boost

(E) Immunofluorescence of T and P
(F) Confocal microscopy of T

(G) Flow cytometry of GFP and CD8

(Reproduced from Ryan and Schell
The Journal of Immunology 2006, 177: p.264)
cells appeared to be either weakly positive or negative for CD8 expression. To verify the CD8+ phenotype of the GFP+ cells present in the tumor stroma, parallel flow cytometric analysis was used to determine the proportion of GFP+ cells that were specific for Tag epitope V. This analysis confirmed that 97% of GFP+ cells isolated from the brain were also CD8+Tet-V+ (Figure 25G). Few GFP+ CD8+ cells were detected in sections from B6 mice (data not shown). These data clearly indicate that TCR-V cells specifically infiltrate choroid plexus tumors following prime/boost immunization.

TCR-V cell adoptive transfer and secondary immunization promotes increased survival of tumor-bearing SV11 mice

Since TCR-V cells penetrated the brain and accumulated at the tumor in SV11 mice that received a prime/boost regimen combined with adoptive transfer, the effect of these cells on tumor growth was investigated (Figure 26). The life spans of SV11 mice treated with the following therapeutic treatments were compared: (i) TCR-V cells only; (ii) TCR-V cells + primary immunization; and (iii) TCR-V cells + prime/boost. Additional control groups included (iv) mice receiving no treatment; (v) mice receiving prime + boost only (no TCR-V cells); and (vi) mice given TCR-V cells and primed and boosted with a cell line expressing Tag in which epitope V has been inactivated (Tag epitope-null). Adoptive transfer of TCR-V cells alone had no effect on survival of SV11 mice (Figure 26). In addition, primary immunization of SV11 mice failed to result in an increase in the lifespan of SV11 mice, despite the infiltration of TCR-V T cells to the tumor site (see Figs 10 and 15). Only after the TCR-V cell adoptive transfer was combined with prime/boost immunization with B6/Tag-V only cells was SV11 survival significantly enhanced. This result indicates that high levels of TCR-V T cell accumulation within the advanced-stage choroid plexus tumors is associated with a significant (p value < 0.0001) increase in survival of SV11 mice.

TCR-V cells do not persist in SV11 mice following secondary immunization

In order to determine whether the presence of TCR-V cells at the tumor site was associated with tumor control in SV11 mice, tumor burden and profile/localization of transferred cells was assessed at the time of death for mice included in the lifespan study. In general,
FIGURE 26

TCR-V Cell Adoptive Transfer and Secondary Immunization Promotes Increased Survival of Tumor-bearing SV11 Mice

The lifespans of tumor-bearing 85-day old SV11 mice treated with the indicated therapeutic treatments were compared. Immunizations (prime and boost) consisted of B6/Tag-V only cells or cells expressing a variant Tag in which epitopes I, II/III, IV, and V were inactivated (Tag epitope-null).

***significantly different from group receiving no treatment by log-rank test.
FIGURE 26

TCR-V Cell Adoptive Transfer and Secondary Immunization Promotes Increased Survival of Tumor-bearing SV11 Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>median survival (days)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>no treatment</td>
<td>102</td>
<td>-</td>
</tr>
<tr>
<td>TCR-V only</td>
<td>101</td>
<td>0.3498</td>
</tr>
<tr>
<td>TCR-V + prime (Tag-V only)</td>
<td>104</td>
<td>0.1039</td>
</tr>
<tr>
<td>TCR-V + prime/boost (Tag-V only)</td>
<td>124</td>
<td>&lt; 0.0001***</td>
</tr>
<tr>
<td>TCR-V + prime/boost (Tag epitope-null)</td>
<td>103</td>
<td>0.1474</td>
</tr>
<tr>
<td>prime/boost only (Tag-V only)</td>
<td>100</td>
<td>0.4965</td>
</tr>
</tbody>
</table>

(Reproduced from Ryan and Schell
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moribund mice were extremely lethargic, exhibited signs of significant neuropathology (ataxia, weakness, paralysis), and bore significant tumor mass in the brain. Additionally, few to no cells were detectable in the spleen. In contrast to the significant accumulation of cells in the brain at 105 days, very few cells were found at the tumor site at later time points, when mice were clearly moribund. An example of the significant decline (12-fold decline) in TCR-V cell frequency in the brain is shown in Figures 27A and B. In contrast to the high frequency detected at 105 days of age, TCR-V cell frequency declined to 5% of total CD8+ cells by 125 days of age – a 12-fold decrease. These results suggest that ultimately the high frequency of TCR-V cells in the brain following adoptive transfer/prime/boost do not persist, correlative with tumor progression. These data imply that continued persistence of TCR-V cells is necessary for tumor control and SV11 survival and that such a prolonged effect cannot be accomplished with the adoptive transfer/prime/boost protocol.

TCR-V cells that accumulate in the brain following secondary immunization are highly susceptible to apoptosis

Since TCR-V cells did not persist in SV11 mice, the possibility that tumor-infiltrating cells are eventually subject to apoptosis was investigated. The spleens and brains of mice that had received TCR-V cell adoptive transfer + prime/boost were assessed at day 105 for tetramer+ cells that stained positive for caspase-3 (an early marker of apoptosis), annexin-V (a late marker of apoptosis), and TUNEL. Camptothecin-treated cells served as a positive control of cell death. Very few Tetramer-V+ splenocytes in B6 or SV11 mice showed up-regulation of apoptotic markers as compared to control. However, a significant population of TCR-V cells isolated from SV11 brains did stain positively for caspase-3 (Figure 27C). A proportion of brain-isolated cells also expressed annexin-V, and TUNEL (data not shown). These data suggest that immunorecessive epitope-V tumor-epitope specific TCR-V cells that accumulate in the brain are eventually susceptible to programmed cell death, and offer a potential explanation for the lack of TCR-V cell persistence in moribund mice.
FIGURE 27

TCR-V Cells Do Not Persist In SV11 Mice Following Secondary immunization, and Are Susceptible to Apoptosis

For mice in the lifespan study, TCR-V cells were transferred into 85 day old SV11 mice followed by prime/boost immunization on the day of transfer and day 7 post-transfer, respectively. Lymphocytes were isolated and analyzed from spleens and brains from moribund mice.

A. A representative example of TCR-V cell frequency in SV11 brains isolated at 105 days of age (20 days post-transfer) and 125 days of age (40 days post-transfer).

B. Frequencies of TCR-V cells in spleens and brains of multiple SV11 mice (8 mice/group) at 105 and 125 days of age.

C. The spleens and brains of SV11 and B6 mice that had received TCR-V cell adoptive transfer + prime/boost were assessed at 105 days of age (40 days post transfer) for tetramer+ cells that stained positive for caspase-3 (an early marker of apoptosis). Camptothecin-treated cells served as a positive control of cell death.
FIGURE 27

A. TCR-V cells isolated from the brains of SV11 mice

105 days old

125 days old

CD8

Tetramer-V

64%

5%

B. TCR-V cells do not persist in SV11 mice following prime/boost immunization

% TCR-V cells/
total CD8+ lymphocytes

105 DAYS

125 DAYS

Age of SV11 Mice at Time of Analysis
C. A large proportion of TCR-V cells isolated from brains of SV11 mice undergo apoptosis

SV11 spleen

B6 spleen (blue: + control)

SV11 brain

red: SV11 spleen  
blue: B6 spleen  
green: SV11 brain  
yellow: camphothecin+ control

CASPASE-3
CONCLUSIONS

From these data, it was concluded that immunorecessive Tag epitope V-specific T-CD8 cells recognize endogenous Tag and gain effector function in vivo. Tag epitope–V specific immunization combined with adoptive transfer promote the accumulation of TCR-V cells at the tumor site, allowing for modest control over tumor progression. However, ultimate morbidity and mortality of SV11 mice correlate with a failure to maintain TCR-V cells at the tumor site – an observation that can at least partially be explained by an increased vulnerability of TCR-V cells to apoptosis.

DISCUSSION

Vaccination approaches that target endogenous self/tumor-reactive T-CD8 cells have met with varying levels of success in a number of tumor models (Colella et al., 2000; Cordaro et al., 2002; Doan et al., 1999; Drake et al., 2005; Ercolini et al., 2003; Lyman et al., 2005; Morgan et al., 1998; Okano et al., 2005; Overwijk et al., 2003). Unfortunately, such self-reactive cells are often subject to peripheral tolerance, limiting their effectiveness against progressing tumors. Additionally, the functional capacity of T cell populations targeting self antigens is often constrained by their intrinsic low-avidity (Cordaro et al., 2002; Theobald et al., 1997), precluding recognition of natural levels of antigen expressed by tumor cells, or resulting in partial delivery of signals downstream of the TCR (de Visser et al., 2001). Thus, limited by the combined effects of peripheral tolerance and/or low avidity, tumor epitope-specific T-CD8 cells have frequently failed to exert control over established tumors (Doan et al., 1998; Lee et al., 1999b; Lyman et al., 2005).

Aware of these limitations, the analyses presented here focused on T-CD8 cell-targeted immunotherapy targeted against a known immunorecessive epitope in mice bearing advanced-stage tumors. Importantly, naïve TCR-V cells were found to recognize epitope V derived from the endogenous wild type Tag in tumor-bearing SV11 mice, demonstrating that this epitope is expressed at significant levels in vivo despite its limited half-life with H2-D^b (Fu et al., 1998). Rapid activation of TCR-V cells induced proliferation, but only low-level accumulation of T cells in the lymphoid organs was observed. Subsequent immunization expanded naïve TCR-V cells
and promoted their initial entry into the brain, although a booster immunization was required to achieve high-level accumulation of functional TCR-V cells in the brains and infiltration into the choroid plexus tumors. Thus, this study reveals that T-CD8 cells specific for epitope V infiltrate advanced-stage tumors, where the accumulation of T cells in the tumor stroma was associated with a delay in tumor progression.

Investigations that have explored T-CD8 cell responses to similar tumor-associated subdominant or immunorecessive epitopes in transplantable tumor models have demonstrated that immunization against specific epitopes can protect against tumor challenge or in some cases lead to the eradication of established tumors (Duraiswamy et al., 2004; Feltkamp et al., 1995; Johnston et al., 1996; Makki et al., 2002; Newmaster et al., 1998). Meanwhile, few studies have focused on T cells that are specifically reactive towards subdominant or immunorecessive tumor epitopes expressed by spontaneously-arising tumors. Previously, Schell et al. found that Tag epitope V specific T-CD8 cells could be expanded from the endogenous T cell repertoire in line 501 SV40 Tag transgenic mice (Schell et al., 2000) and line SV11 mice (Schell, 2004). However, the effect on tumor growth was not addressed. Likewise, using the TRAMP mouse model of prostate cancer, Grossmann et al (Grossmann et al., 2001) reported the ability to recruit T-CD8 cells against the SV40 Tag-V epitope, but did not assess the effect on tumor progression. Singh et al (Singh et al., 2005) recently described the discovery of subdominant T-CD8 cell epitopes within the HER-2/neu antigen that could induce an antitumor response in mice that express HER-2/neu (Singh and Paterson, 2006), and the ability of immunization to control spontaneous mammary tumors in these mice was only recently described (Singh and Paterson, 2007). Thus, at the time the studies presented here were performed, the effect of T-CD8 cells specific for an immunorecessive tumor epitope on spontaneous tumor progression had not been explored.

In order for an epitope to be effectively targeted in vivo, it must be efficiently presented for recognition by the responding T-CD8 cell. This requirement is particularly important for immunorecessive epitopes, which might be limited by antigen processing or presentation. Two lines of evidence indicate that epitope V is presented in vivo from wild type Tag in SV11 mice. First, naïve TCR-V T cells were activated and proliferated in the lymphoid organs following transfer into SV11 mice, although expansion was limited. This finding is consistent with recently published data in which TCR-V cells transferred into B6 hosts are activated, but undergo minimal expansion upon immunization with wild type Tag-transformed cells. The findings from
Otahal et al. (Otahal et al., 2005) illuminated some of the mechanisms that contribute to the immunorecessive phenotype of Tag epitope V. The weak response in B6 mice was attributed to inefficient cross-presentation of Tag-V epitope, leading to poor priming of naïve epitope V-specific T cells (Otahal et al., 2005). This observation is likely due to the formation of unstable H-2D<sup>b</sup>/epitope V complexes (Fu et al., 1998), which may fail to provide a prolonged stimulus (van Stipdonk et al., 2001). Additionally, the presence of T cells specific for immunodominant epitopes that compete for access to the same antigen-presenting cells may contribute toward the immunorecessive nature of Tag epitope-V specific T-CD8 cells (Otahal et al., 2005). In SV11 mice, T-CD8 cell precursors specific for the three most immunodominant epitopes are absent from the residual repertoire (Schell et al., 1999). Therefore, the limited accumulation of TCR-V cells following exposure to endogenous Tag might partially be due to poor cross-presentation of the epitope. Rapid dissociation of epitope V/D<sup>b</sup> complexes from the cell surface may result in premature disruption of antigen engagement, preventing efficient T cell expansion (van Stipdonk et al., 2001). Whether epitope V/D<sup>b</sup> complexes on APC are limiting in SV11 mice remains to be determined.

The second finding indicating that epitope V is presented in vivo in SV11 mice was demonstrated by the accumulation of TCR-V cells specifically in the brains and choroid plexus tumors following the prime and boost immunization. Other studies that were primarily performed using transplantable tumors expressing foreign antigen have also observed tumor antigen specific accumulation of activated T cells at the tumor site (Kedl and Mescher, 1997; Plautz et al., 1997; Yu et al., 2004; Yu et al., 2005). By contrast, it has been reported that under tolerizing conditions, immunodominant self-epitope specific T-CD8 cells do not readily penetrate transplanted tumors that express self-antigen (Lyman et al., 2005; Overwijk et al., 2003). The results presented here show that in the context of self-antigen expression, functionally competent Tag-V-specific T cells accumulate within the tumor, indicative of specific recognition of this epitope in vivo.

A central question addressed in this study is whether high avidity T-CD8 cells responding to an immunorecessive self-antigen epitope would be affected by tolerance in much the same manner as T-CD8 cells responding to immunodominant epitopes. Such mechanisms could include anergy and/or deletion. The data demonstrate that TCR-V cells acquire effector function in response to the endogenous Tag. It remains possible that prolonged exposure to endogenous Tag renders TCR-V cells destined for future deletion since these cells are not
retained at detectable levels unless the mice are immunized. Similar observations have been made with TCR transgenic T cells responding to immunodominant tumor epitopes upon transfer into tumor-bearing mice (Lyman et al., 2005; Ohlen et al., 2001; Overwijk et al., 2003). The findings presented here using TCR transgenic T cells imply that higher avidity endogenous epitope V specific T-CD8 cells that survive negative selection in SV11 mice might additionally be subject to tolerance upon recognition of and activation by persistent Tag in the peripheral tissues, thereby contributing to the low levels of functional epitope V specific T cells available for recruitment in tumor-bearing mice (Schell, 2004). Whether the endogenous epitope V-specific T cells isolated from SV11 mice have avidities similar to the TCR-V T cells has not been determined. If so, their lasting presence might require continued export of new cells from the thymus (Tanchot and Rocha, 1997).

Immunization of SV11 mice extended the expansion phase of TCR-V cells, but did not increase the magnitude to that observed in B6 mice. There are several possible explanations for the lower total accumulation of TCR-V cells in SV11 mice versus B6 mice following immunization. First, kinetic analysis revealed that adoptively transferred cells can be triggered by epitope V derived from endogenous Tag 24-48 hours before the effects of immunization are realized. A similar lag in response to immunization has been observed in other systems (Estcourt et al., 2005; Feuerer et al., 2003; Koido et al., 2002) and may correlate with the time required for antigen presenting cells to localize to secondary lymphoid organs (Randolph et al., 2005). Thus, T-CD8 cells that have encountered endogenous Tag may initially be refractory to rapid re-activation by exogenous Tag delivered on the day of adoptive transfer due to the induction of activation-induced inhibitory pathways (Riley and June, 2005).

Alternatively, there could be fewer T-CD8 cells available for exposure to Tag derived from immunization if the cells have migrated to a more distant tissue. In fact, it was found that TCR-V T cells accumulated rapidly in the CLN following transfer into SV11 mice, distant from the immunization site in the peritoneal cavity. Another possibility is that some of the transferred TCR-V T cells are irreversibly inactivated by recognition of the endogenous Tag, due to the immediate and persistent expression of Tag. Immunization of SV11 mice provides an additional source of antigen outside of the CLN that might convey a qualitatively different signal from that delivered by antigen draining from the tumor site. It has been postulated that the nature of the initial encounter with APC can program the activation, expansion, function, and ultimate survival of naïve T-CD8 cells (Gett et al., 2003; Heath and Carbone, 2001b). Potentially, an APC that
displays endogenously-derived Tag-V originating from the brain might deliver a tolerogenic signal to the naive TCR-V cell it engages. Meanwhile, an APC that cross-presents exogenously-derived Tag-V might confer a contrasting survival signal. Whether direct interaction with B6/V-only Tag cells may rescue TCR-V cells that have been partially activated by endogenous Tag remains unknown.

These data suggest that the migration of TCR-V cells to the brains of SV11 and B6 mice is not initially antigen dependent following primary immunization. Rather, the initial migration into the brain reflects the presence of activated cells in the periphery. A similar observation was made previously for T-CD8 cells responding to the dominant Tag epitope IV (Schell and Tevethia, 2001). Entry into the central nervous system has been reported to require the expression of activation molecules such as CD38 (Graesser et al., 2002; Qing et al., 2001) and CD43 (Onami et al., 2002) in addition to the up-regulation of specific homing receptors (Fabry et al., 1992; Hickey et al., 1991). Extravasation is subsequently dependent upon interaction of these receptors with their cognate binding partners on the brain vasculature, including I-CAM, V-CAM, PE-CAM, and P-selectin. Preliminary data from these studies has shown that a subset of TCR-V cells recovered from the spleens of immunized SV11 and B6 mice express CD38, the lymphocyte receptor for the endothelial ligand PE-CAM. Furthermore, a homogeneous population of brain-isolated TCR-V cells up-regulated this homing molecule.

In this study, the booster immunization drove a dramatic accumulation of TCR-V cells in the brains of SV11 but not B6 mice. A possible explanation for this finding is that booster immunization leads to an initial expansion of antigen experienced TCR-V cells in the peripheral lymphoid organs, as evidenced by a dramatic increase in the frequency of TCR-V T cells in the spleens of B6 mice. In SV11 mice, these cells might rapidly migrate to and accumulate in the large choroid plexus tumors. In B6 mice, these cells likely migrate through the brain and back into the circulation in the absence of specific antigen. Whether the large accumulation of TCR-V T cells in the brains of SV11 mice that received a booster immunization is due to the influx of cells from the periphery or represents local expansion within the tumor remains to be determined.

The effect of the booster immunization in SV11 mice was quite dramatic compared to the effect of the primary immunization despite the presence of a large number of precursor cells within the lymphoid organs at the time of initial immunization. The data indicate that the majority
of transferred TCR-V cells are rapidly triggered following exposure to the endogenous Tag in SV11 mice and most likely remain in an activated state 24-48 hours after transfer. Thus, they might be unable to efficiently respond to the B6/V-only Tag immunization administered on the same day. However, following contraction at day 5, they demonstrate enhanced capability of responding to the B6/V-only Tag booster administered on day 7. Suboptimal activation of T-CD8 cells has been shown to occur in vivo, leading to a T cell population that, while antigen experienced and capable of IFN-γ production, remains undivided (Auphan-Anezin et al., 2003). However, this incompletely differentiated cell population responded to secondary challenge. Therefore, TCR-V cells that receive a suboptimal signal after stimulation by the endogenous Tag might become fully activated and able to migrate to the tumor upon delivery of the day 7 booster immunization.

Despite the vigorous enhancement of epitope V-specific T-CD8 cells at the tumor site and significant increase in survival, all mice eventually succumbed to tumor burden. Evidence from these studies indicate that the frequency of tumor resident TCR-V cells declines significantly in primed and boosted SV11 mice by the time of death, and that a large proportion of brain-infiltrating TCR-V cells eventually undergo apoptosis. Thus it is possible that TCR-V cells are eventually subject to the same tolerogenic mechanisms as those observed in immunodominant epitope-specific T cell models. A similar transient effect was observed in SV11 mice following the activation of adoptively transferred normal B6 spleen cells against the immunodominant epitope Tag IV by specific immunization (Schell et al., 1999). Common obstacles to T-CD8 cell-mediated tumor immunotherapy include alterations at the tumor site itself, including (i) the presence of T regulatory cells, (ii) tumor cell down-regulation of MHC class I expression, (iii) the emergence of tumor cell escape variants that cease to express the epitopes recognized by their cognate tumor-reactive T cells, and (iv) production of T cell suppressive cytokines by the tumor (Blohm et al., 2002; Khong and Restifo, 2002). Expression of the anti-inflammatory cytokine TGF-β has been reported in SV11 mice with progressing tumors (Roy et al., 2000). Therefore, it is possible that eventually TCR-V cells are rendered non-functional by the tumor microenvironment. Thus, it will be important to address the state of the tumor microenvironment in order to investigate whether similar mechanisms of immune evasion occur in response to immunorecessive epitope V-specific T-CD8 cells.
CHAPTER V

COMBINATORIAL IMMUNOTHERAPY TARGETED AGAINST BOTH ENDOGENOUS AND EXOGENOUS FORMS OF T ANTIGEN EPITOPE V PROMOTES LONG-TERM PERSISTENCE OF TCR-V CELLS AT THE TUMOR SITE AND SIGNIFICANTLY PROLONGS SURVIVAL OF SV11 MICE

GOALS

It was proposed that long-term persistence of TCR-V cells at the tumor site was required for prolonged control over SV11 tumor progression. The objective of specific aim #2 was to identify potential strategies for enhancing the in vivo TCR-V cell-mediated anti-tumor effect, specifically approaches that promoted maintenance of TCR-V cells in SV11 tumors. This was accomplished by investigating the response of TCR-V cells to different immunotherapeutic regimens involving adoptive transfer into SV11 mice, specifically optimization of the in vivo activation against the endogenous Tag and variations in the kinetics and cell-type used for exogenous cellular immunization. The effects of immunotherapy were determined by the assessment of TCR-V cell expansion, functionality, tumor trafficking properties, persistence and effect on SV11 survival.

OVERVIEW

The response of TCR-V cells to both endogenous Tag as well as exogenous (cellular immunization) Tag was explored even further, with a focus on approaches that drive TCR-V cell expansion and persistence. Additionally, strategies aimed at augmenting epitope V-targeted adoptive immunotherapy of SV11 mice bearing advanced stage tumors were investigated. It was found that the cellular source of antigen, mode of antigen presentation, and timing of immunization played critical roles in elicitation of the TCR-V cell response in vivo. Additionally the data demonstrate that adoptive T-CD8-mediated immunotherapy against spontaneous SV11 choroid plexus tumors was optimized by enhanced sensitization against the endogenous tumor antigen and carefully-timed immunization. Furthermore, combinatorial approaches that coupled therapeutic modalities based on TCR-V cell activation by both endogenous and exogenous Tag synergistically enhanced both the maintenance of tumor-infiltrating TCR-V cells as well as SV11 survival. These findings implicate the potential for similar combinatorial therapeutic approaches in cancer patients.
INTRODUCTION

The environment in which T-CD8 cells engage tumor antigen peptide:MHC class I complexes often dictates the magnitude and effectiveness of the anti-tumor response (Fuchs and Matzinger, 1996). Since many tumors lack co-stimulatory molecules, the most likely mode of T cell priming is thought to involve professional APCs such as dendritic cells that cross-present tumor-derived antigens in tumor draining lymph nodes (Heath and Carbone, 2001b; Huang et al., 1994; Norbury and Sigal, 2003; Ochsenbein et al., 2001). According to the presence or absence of immuno-stimulatory conditions during interaction of a naïve T-CD8 cell with a DC, cross-presentation of tumor antigens may lead to either robust activation or tolerance (Belz et al., 2002; Heath and Carbone, 2001a, b; Huang et al., 1994; Kurts et al., 1996; Nguyen et al., 2002; Sotomayor et al., 2001). The capacity for a DC to properly activate a T-CD8 cell corresponds with the maturation state of the DC, characterized by enhanced expression of co-stimulatory molecules including MHC class I and II molecules and CD80/CD86 (Banchereau et al., 2000; Lanzavecchia and Sallusto, 2001; Steinman et al., 2003). DCs may also cross-present antigen in the absence of co-stimulation, leading to deletion or anergy of antigen-specific T cells (Bansal-Pakala et al., 2001; Chen et al., 1999; Cooper et al., 2002; Diehl et al., 2002; Hendriks et al., 2000; Hernandez et al., 2001; Ohlen et al., 2002; Toes et al., 1998b). Thus, the state of an APC cross-presenting tumor antigen critically determines the potential to successfully elicit an effector T cell response in a tumor-bearing host (Albert et al., 2001; Blankenstein and Schuler, 2002; Shortman and Heath, 2001).

Following adoptive transfer into SV11 mice, TCR-V cells demonstrated early recognition of endogenous Tag in the tumor-draining CLN (Figure 18). However, despite activation by endogenous Tag and initial proliferation, TCR-V cells displayed an incompletely activated phenotype, did not significantly accumulate, and were not therapeutic. Under steady-state conditions, LN-resident DCs generally manifest an immature phenotype, and such DCs may feasibly induce anergy when presenting tumor antigen that drains specifically to a LN (Haanen et al., 2000). Irrespective of maturation state, DCs may induce T-CD8 cell division following only 2 hours of contact. However, full programming of effector and memory responses requires at least 24 hours of engagement (Kaech and Ahmed, 2001; Kaech et al., 2002; van Stipdonk et al., 2001). Although brief periods of stimulation may result in proliferation, T cells frequently undergo abortive proliferation and demonstrate limited survival due to failure to divide in
response to IL-7 or IL-15 (Auphan-Anezin et al., 2003; Cao et al., 1995). However, sufficient stimulation enhances cytokine responsiveness, effector function, and ultimate T-CD8 cell survival (Gett et al., 2003; van Stipdonk et al., 2003). Prolonged T cell-DC contact and optimal T-CD8 cell activation may depend upon proper maturation of the DC, facilitated by stimulating signals delivered by TLR ligands, inflammatory cytokines and/or ligation of the CD40 receptor (Banchereau et al., 2000; Banchereau and Palucka, 2005; Banchereau and Steinman, 1998; Dhodapkar et al., 2001; Dhodapkar et al., 1999; Lambolez et al., 2002; Lanzavecchia, 1998). It was therefore postulated that in the absence of immunization, cross-presentation of tumor-derived endogenous Tag in the CLN was insufficient for complete TCR-V cell activation and potentially delivered a tolerizing signal to TCR-V cells.

One of the challenges faced by APCs that cross-present self/tumor antigens is the absence of inflammatory stimuli, which often renders APCs incapable of initiating a potent anti-tumor response, and results in tolerance toward the growing tumor (Fuchs and Matzinger, 1996; Matzinger, 1994). Hindrance to the development of optimal T-CD8 responses can be overcome via manipulation of the maturation status of the APC in vivo, whereby T cell help is recapitulated by administration of an agonistic mAb to CD40 (reviewed in (Mackey et al., 1998a; Quezada et al., 2004; Toes et al., 1998a; van Kooten and Banchereau, 2000).) This treatment mimics engagement of CD40 on APCs by CD154 (CD40L) on activated CD4+ T cells, leading to APC up-regulation of co-stimulatory molecules and increased cytokine production (Bennett et al., 1998; Mackey et al., 1998b; Ridge et al., 1998; Schoenberger et al., 1998). Immunotherapeutic strategies based on CD40 pre-conditioning have demonstrated significant enhancement of the T-CD8 response against both transplantable and spontaneous tumors (Diehl et al., 1999; French et al., 1999; Mackey et al., 1998a; Staveley-O'Carroll et al., 2003; Todryk et al., 2001; van Mierlo et al., 2004; van Mierlo et al., 2002). If non-stimulatory or tolerizing DCs in the CLNs of SV11 precluded optimal activation, tumor trafficking and survival of TCR-V cells, pre-conditioning SV11 mice with mAb to CD40 prior to adoptive transfer posed a tempting option for augmenting TCR-V cell responses to SV11 tumors in vivo.

A puzzling finding from initial studies reported in Chapter IV concerned the weak response to primary immunization administered only on the day of adoptive transfer compared to priming followed by secondary immunization on day 7 (Figure 22). In B6 mice, TCR-V cells were not activated by Tag-V derived from the immunizing cell line (B6/V-only Tag cells) until 48 hours post immunization (Figure 18). This result was attributed to the time required for antigen
uptake, processing and presentation by APCs. However, in SV11 mice, endogenous Tag is presumably immediately available for presentation to transferred TCR-V cells, as cells have already undergone multiple rounds of proliferation by 48 hours post transfer (Figure 18). Thus, the majority of TCR-V cells engaged by tumor-derived Tag in the CLN might be refractory to exogenous immunization delivered coincident with adoptive transfer. It is feasible that upon contraction from stimulation by endogenous Tag, TCR-V cells regain potential responsiveness to immunization, at which point the exogenous antigen may have already been cleared. Additionally, Tag-V peptide/MHC complexes, which are known to be relatively unstable, may have already fallen apart (Fu et al., 1998). The idea that for a window of time post transfer into SV11 mice, TCR-V cells responding to endogenous Tag are unable to respond to immunization was supported by the dramatic reaction following secondary immunization administered 7 days post transfer. This resulted in accumulation of TCR-V cells in SV11 tumors (Figure 22), correlative with prolonged SV11 survival (Figure 16). It was thus proposed that timing of immunization may play a significant role in the responsiveness of TCR-V cells adoptively transferred into the Tag-expressing environment of tumor-bearing SV11 mice.

Otahal et al. (Otahal et al., 2005) recently demonstrated inefficiency in cross-presentation of Tag-V epitope, and showed that in B6 mice, both cross and direct presentation are necessary in order to achieve maximal expansion of TCR-V cells by Tag V-only cellular immunization. However, this requirement in SV11 mice that express endogenous Tag remained unknown. Additionally, while TCR-V cells recognize epitope V derived from endogenous Tag in SV11 mice, the potential for TCR-V cells to respond to cellular immunization with wild-type Tag in SV11 mice remained undetermined. The mode of Tag V presentation and the source of Tag V derived from cellular immunization (wild type vs B6/Vonly-Tag) may significantly alter the effect of immunization on TCR-V cells in SV11 mice.

The uncertain requirements for optimal responsiveness to immunization and the vast potential for improving upon TCR-V cell mediated immunotherapy in SV11 mice precipitated the following questions: (i.) What is the optimal time for exogenous immunization following TCR-V cell adoptive transfer into SV11 mice bearing advanced-stage tumors? (ii.) Do TCR-V cells respond to wild type Tag derived from cellular immunization? (iii.) What mode(s) of presentation are necessary to elicit optimal expansion of TCR-V cells in SV11 mice following cellular immunization? (iv.) Can the initial TCR-V response to endogenous Tag in SV11 mice be enhanced in vivo?
In order to better understand how the response against an immunorecessive tumor epitope might be prolonged within the setting of the tumor-bearing host, the subsequent studies investigated the impact of the following immunotherapeutic manipulations on the response of TCR-V cells and control of tumor progression following transfer into SV11 mice bearing advanced-stage tumors:

(i.) Variations in the the timing of Tag-V specific immunization
(ii.) Adoptive transfer of pre-immune in vivo-activated TCR-V cells
(iii.) Immunization with cells expressing full length wild-type Tag
(iv.) Immunization with Tag V-only cells that are incapable of direct presentation
(v.) Repeated booster immunization with Tag V-only cells
(vi.) Sensitization against endogenous Tag by pre-conditioning with anti-CD40 mAb
(vii.) Combinatorial therapy incorporating multiple treatment modalities
RESULTS

Timing of immunization against Tag epitope V is critical for optimal expansion of TCR-V cells in SV11 mice

The observation that administration of primary immunization on the same day of adoptive transfer resulted in suboptimal accumulation of TCR-V cells in SV11 mice led to the hypothesis that immediate activation by endogenous Tag rendered TCR-V cells refractory to responding to exogenous Tag, thereby compromising the effect of immunization. Thus, it was proposed that immunization at a later time point, following contraction from initial activation against endogenous Tag, would augment the efficiency of the immunization. Experiments were designed in an attempt to identify the time of immunization necessary for promoting maximal expansion of adoptively transferred TCR-V cells in SV11 mice.

Before deciding on the most advantageous time for immunization, it was necessary to determine how long TCR-V cells persisted in SV11 mice in the absence of immunization. To address this question, TCR-V cells were adoptively transferred into 85 day-old SV11 mice, and groups of mice were analyzed at weekly intervals (day 7, 14, 21, and 28) for TCR-V cell frequency (Figure 28A). It was observed that maximum frequency was achieved on day 7 post transfer (3.8% total CD8+ cells). TCR-V cell numbers subsequently declined, reaching 1.5% of CD8+ cells at one month following transfer (Figure 29A). By comparison, TCR-V baseline frequency remained stable and similar to engrafted levels (1.9%-2.3% of CD8+ cells) in unimmunized B6 mice throughout the 4-week time course (Figure 29A). The progressive loss of TCR-V cells in SV11 mice suggested that these cells were deleted as a result of tolerance to the endogenous Tag.

In order to assess TCR-V cell responsiveness to exogenous immunization post transfer, mice were immunized at these different time points. TCR-V cells were labeled with CFSE and adoptively transferred into 85 day-old SV11 mice. Groups of mice were immunized with B6/V-only Tag cells at 0, 7, 14, or 21 days following transfer. A control group received no immunization. Each group was then analyzed 7 days post-immunization. Spleens were harvested and assessed for the frequency and activation status of TCR-V cells by staining for
FIGURE 28

Protocol for Timed Immunization of SV11 Mice Following TCR-V Cell Adoptive Transfer

A. In order to determine how long TCR-V cells persisted in SV11 mice in the absence of immunization, TCR-V cells were adoptively transferred into 85 day-old SV11 mice, and groups of mice (3 mice/group) were assessed at weekly intervals (day 7, 14, 21, and 28) for TCR-V cell frequency in the spleen.

B. In order to determine optimal responsiveness of TCR-V cells to immunization, TCR-V cells were labeled with CFSE and adoptively transferred into 85 day-old SV11 mice or B6 mice. Groups of mice (3 mice/group) were immunized with B6/Tag-V only cells at weekly intervals, including day 0, 7, 14, or 21 following transfer. A control group received no immunization. Each group was then analyzed 7 days post-immunization to assess frequency and activation of TCR-V cells in the spleen.
A.

![Diagram A](image)

**FIGURE 28**

TCR-V cell adoptive transfer into SV11 or B6 mice

**ANALYSIS**
Isolate TCR-V cells from spleen

Day 0

Day 7

Day 14

Day 21

Day 28

B.

Adoptive transfer of naïve TCR-V cells + varied timing of immunization

**Adoptive transfer**
(day 0)

**Ex vivo analysis**

**Tag-V specific immunization**

**AT**

**Prime**

**Analysis**

None

Day 0

Day 7

Day 14

Day 21

Day 28
FIGURE 29

Timing of Immunization Against Tag Epitope-V is Critical For Optimal Expansion of TCR-V Cells in SV11 Mice

TCR-V cells Do Not Persist in SV11 Mice In the Absence of Immunization

A. In order to determine how long TCR-V cells persist in SV11 mice in the absence of immunization, TCR-V cells were adoptively transferred into 85 day-old SV11 mice, and groups of mice (3 mice/group) were analyzed at weekly intervals (day 7, 14, 21, and 28) for TCR-V cell frequency in the spleen by staining for CD8 and Tag-V tetramer.

Immunization On Day 7 Post Transfer Promotes Optimal Accumulation of TCR-V Cells in SV11 Mice

B. In order to determine optimal responsiveness of TCR-V cells to immunization, TCR-V cells were labeled with CFSE and adoptively transferred into 85 day-old SV11 mice or B6 mice. Groups of mice (3 mice/group) were immunized with B6/Tag-V only cells at weekly intervals, including day 0, 7, 14, or 21 following transfer. A control group received no immunization. Each group was then analyzed 7 days post-immunization. Spleens were harvested and assessed by tetramer analysis for the frequency (% of CD8+ cells) of TCR-V cells by staining for CD8 and Tag-V tetramer. Gated populations of CD8+Tet-V+ cells were assessed for activation status (CD44) and proliferation (CFSE dilution). Separate groups of unimmunized mice were analyzed at day 7, 14, 21 and 28 to determine baseline frequency of TCR-V cells at the time of immunization. These frequencies are indicated in parentheses to indicate the effect of immunization on the baseline frequency at each time point.

C. The frequency of TCR-V cells is represented as the absolute number of splenocytes for each experimental group.
FIGURE 29

A. Baseline frequency of TCR-V cells in the absence of immunization

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>3.8%</td>
<td>2.7%</td>
<td>2.1%</td>
</tr>
<tr>
<td>Tag-V tetramer</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>2.3%</td>
<td>1.9%</td>
<td>2.2%</td>
</tr>
<tr>
<td>Tag-V tetramer</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Timing of Immunization is Critical for Optimal TCR-V Cell Accumulation in SV11 mice

<table>
<thead>
<tr>
<th>Time of immunization</th>
<th>SV11 gated on CD8+Tet-V+ cells</th>
<th>B6 gated on CD8+Tet-V+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>3.5% (3.7%)</td>
<td>2%</td>
</tr>
<tr>
<td>day 0</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>30% (3.7%)</td>
<td>25% (1.7%)</td>
</tr>
<tr>
<td>day 14</td>
<td>5.7% (2.2%)</td>
<td>23% (2.0%)</td>
</tr>
<tr>
<td>day 21</td>
<td>2.3% (1.7%)</td>
<td>20% (1.9%)</td>
</tr>
</tbody>
</table>
FIGURE 29

C. Timing of Immunization is Critical for Optimal TCR-V Cell Accumulation in SV11 mice

![Bar graph showing the absolute number of Tag-V+ cells in the spleen over different time points of immunization. The graph compares SV11 and B6 strains.](image-url)
CD8 and Tag-V tetramer. Gated populations of CD8+Tet-V+ cells were assessed for activation status (CD44) and proliferation. Separate groups of unimmunized mice were analyzed at day 7, 14, 21 and 28 to determine baseline frequency of TCR-V cells at the time of immunization (Figure 28B).

TCR-V cell frequencies in unimmunized SV11 mice were similar to levels observed previously (Figure 21a), whereby the maximal frequency observed on day 7 (3-4% of CD8+ cells) subsequently declined by day 28 post transfer (Figure 29B parentheses). Additionally, in B6 mice, the engrafted TCR-V cell population in the absence of immunization remained near 1-2 % of CD8+ cells during the 4-week course (Figure 29B parentheses). In response to the administration of immunization at weekly intervals, optimal expansion of TCR-V cells in SV11 mice was observed when immunization was delivered on day 7 post transfer (30% of CD8+ splenocytes) (Figure 29B). These cells expressed high levels of CD44 and had undergone multiple rounds of proliferation as shown by loss of CFSE. Immunization administered at day 0 or at later time points failed to induce this same level of accumulation. Interestingly, when immunization was delivered on day 0, some undivided cells were still detected, indicating that these cells had not yet encountered antigen. However, when immunization was delivered at later time points (days 14 and 21), all TCR-V cells had lost CFSE label, suggesting that following activation by endogenous Tag and subsequent proliferation, TCR-V cells were deleted, since cells did not accumulate.

In B6 mice, robust expansion was also observed when immunization was delivered on day 7 post transfer (25% of CD8+ splenocytes) (Figure 29B). However, similar frequencies were achieved when immunization was delivered at later time points (23% of CD8+ cells at day 14 and 20% of CD8+ cells at day 21) (Figure 29B). Additionally, these cells had proliferated extensively, providing evidence that TCR-V cells retained the potential to respond optimally to exogenous immunization. A comparable trend was observed when these results were analyzed as total number of TCR-V splenocytes, with TCR-V cells peaking at 1.3x10^6 cells in SV11 mice following day 7 immunization (Figure 29C). These data indicate that timing of immunization is critical for maximal TCR-V cell expansion in SV11 mice, with day 7 being optimal, and suggest that prolonged exposure to endogenous Tag ultimately tolerizes TCR-V cells, rendering them unresponsive to immunization.
Immunization on day 7 promotes accumulation of functional TCR-V cells at the tumor site in SV11 mice

Since immunization of SV11 mice on day 7 post transfer was essential for maximal expansion of TCR-V cells in the spleen, it was important to determine whether this cell population harbored functional capacity and trafficked to the tumor site. To address this question, TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Groups of mice received (i) no immunization; (ii.) immunization on the day of transfer (day 0); or (iii.) immunization on day 7 post transfer. Splenocytes and brain lymphocytes were assessed for TCR-V cell frequency and for production of IFN\(\gamma\) in response to Tag-V peptide stimulation \textit{ex vivo} via intracellular cytokine assay. Once again, immunization on day 7 post transfer resulted in dramatic accumulation of TCR-V cells in the spleens (28% of CD8+ cells) (\textbf{Figure 30}). Additionally, day 7 immunization promoted high level infiltration and accumulation of TCR-V cells in the brain (50% of CD8+ cells) by day 14. Furthermore, a population of CD8+ cells in both the spleen (20%) and brain (32%) produced Tag-V specific IFN\(\gamma\), indicating that a significant proportion of TCR-V cells that accumulate in SV11 mice following day 7 immunization are functional. As previously shown (Figure 22 and Table 1) (Ryan and Schell, 2006), immunization at day 0 failed to promote high-level TCR-V cell accumulation in the brain (\textbf{Figure 30}). These data further support the finding that proper timing of immunization post transfer is crucial for eliciting an optimal response in SV11 mice, and demonstrate that day 7 immunization delivers signals that promote accumulation of functional T cells specific for Tag V at the tumor site, without the need for a booster.

Immunization on day 7 promotes accumulation of actively proliferating TCR-V cells at the tumor site, but ultimately TCR-V levels are not maintained

Studies in Chapter I indicated that a prime and boost regimen of immunization delivered on days 0 and 7 respectively, promoted high-level accumulation of TCR-V cells within SV11 tumors (Figure 22 and Table I) (Ryan and Schell, 2006). However, ultimately these cells did not persist – an effect attributed in part to elevated susceptibility to apoptosis (Figure 27). These observations drove the interest to learn how long TCR-V cells persisted following day 7 immunization. TCR-V cells were transferred into 85-day old SV11 mice and mice were
Immunization on Day 7 Promotes Accumulation of Functional TCR-V Cells at the Tumor Site in SV11 Mice

The TCR-V cell population expanded with day 7 immunization was assessed for functional capacity and ability to traffic to the tumor site. TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Groups of mice (3 mice/group) received (i) no immunization; (ii.) immunization on the day of transfer (day 0); or (iii.) immunization on day 7 post transfer. Splenocytes and brain lymphocytes were isolated and assessed 7 days later for TCR-V cell frequency in the spleen and brain by MHC I tetramer staining, and for production of IFNγ in response to Tag-V peptide stimulation ex vivo via intracellular cytokine assay. (% = % of tetramer-V+ or IFNγ+ /total CD8+ cells)
FIGURE 30

Immunization on Day 7 Promotes Accumulation of Functional TCR-V Cells at the Tumor Site in SV11 Mice
immunized 7 days later. Mice were assessed at various time points post immunization to measure the frequency of TCR-V cells in the spleen, CLN, and brain (Figure 31A). In order to determine whether cells were actively proliferating at these time points, each group of mice was injected with BrdU for 2 days prior to analysis, allowing for detection of the percentage of TCR-V cells that had divided during the last 48 hours (Figure 31B and C).

In B6 mice, TCR-V cells demonstrated kinetics reflective of normal T cell expansion and contraction. TCR-V cell numbers peaked at 2.2x10^6 cells in the spleen at day 7 post-immunization (Figure 31A). This was preceded by 28% of total TCR-V cells proliferating in the spleen on day 4 (Figure 31C). Similar kinetics were observed in the CLN of B6 mice, with maximum frequency reaching 8.2x10^3 cells on day 7 (Figure 31A), corresponding to 7% total TCR-V cells that had divided between day 5 and day 7 (Figure 31C). A population of TCR-V cells was transiently detected in B6 brains at day 7, but high levels of TCR-V cells never accumulated (Figure 31A).

In SV11 mice, similar kinetics were observed for TCR-V cells in the spleen. TCR-V cell frequency peaked at day 7 (1.8x10^6 cells) (Figure 31A), and the highest percentage of dividing cells was detected at day 4, similar to B6 mice (Figures 31B and C). In the CLN of SV11 mice, TCR-V cells were already detectable at the time of immunization (8.2x10^3 cells) (Figure 31A), and 20% of these TCR-V cells were proliferating, presumably due to activation by endogenous Tag (Figure 31C). Surprisingly, this frequency decreased in the CLN following immunization, falling to 2.1x10^3 cells at day 21. This decline in TCR-V cell frequency in the CLN corresponded with a sharp increase in TCR-V cells detected in the brain between day 2 and day 4 post-immunization (Figure 31A). While TCR-V cell frequency peaked at 1.0x10^6 cells detected on day 7, high levels of TCR-V cells were maintained at the tumor site, even at day 21 (6x10^4 cells) (Figure 31A).

The in vivo incorporation of BrdU enabled the determination of whether these TCR-V cells were being maintained by active proliferation, or whether they represented an accumulation of non-dividing cells. A large fraction of brain-resident TCR-V cells had recently divided before the day 4, 7, and 14 analysis time points, with the percentage of proliferating TCR-V cells ranging between 50-60% (Figures 31B and C). However, the population of actively dividing TCR-V cells in the brain ultimately declined to 25% by day 21 post-immunization (Figure 31C) and was accompanied by a modest decrease in total TCR-V cells at
The kinetics of the TCR-V cell response following day 7 immunization were assessed, specifically when activated TCR-V cells gain access to the brain. TCR-V cells were adoptively transferred into 85 day-old SV11 or B6 mice. At 7 days post transfer, mice were immunized with B6/V-only Tag cells.

A. One group of mice was analyzed prior to immunization in order to assess the base-line frequency of TCR-V cells before priming. Groups of mice (3 mice/group) were analyzed at 5 time points post immunization: 2 days, 4 days, 7 days, 14 days, and 21 days post immunization - to assess frequency of TCR-V cells in the spleen, CLN, and brain by MHC I tetramer staining. Error bars indicate standard deviation of the mean.

B. The kinetics of the TCR-V cell proliferative response following day 7 immunization were assessed. The same groups of mice were injected i.p. every 12 hours with 4 doses of 1 mg/mL BrdU 2 days prior to sacrifice in order to label proliferating cells. TCR-V cells isolated from the spleens, CLNs, and brains were analyzed for BrdU incorporation by CD8+/Tetramer-V+ cells. Representative BrdU histograms gated on CD8+/Tetramer-V+ cells of a mouse assessed at 4 days post-immunization are shown.

C. Proliferative data from all mice in the study is represented as the % of TCR-V cells staining positive for BrdU in each organ at each analytic time point. Error bars indicate standard deviation of the mean.

D. In order to address long-term persistence of TCR-V cells following primary immunization administered on day 7, a group of 6 SV11 mice was analyzed 33 days following immunization, at 125 days of age to assess TCR-V frequency remaining in the spleen and brain. Representative dot plots for CD8 and Tetramer-V stained cells are shown. (% = % Tet-V+ cells /total CD8+ cells; parentheses indicate absolute # of Tet-V+ cells).
FIGURE 31

A. Immunization on day 7 promotes trafficking of TCR-V cells from the CLN to the brain

B. TCR-V cells that accumulate in the brain following day 7 Immunization are actively dividing

C. High levels of TCR-V levels do not persist at the tumor site following immunization on day 7

D. TCR-V cell frequency in 125 day old SV11 mice (33 days post day 7 immunization)
this time point (Figure 31A). These results suggest that while day 7 immunization promotes TCR-V cell accumulation at the tumor site, the effect is transient. However, the brain-infiltrating TCR-V cell population is more long-lived compared to immunization delivered on day 0 (Figure 27).

In order to address long-term persistence of TCR-V cells following primary immunization administered on day 7, a group of SV11 mice was analyzed 33 days following immunization, at 125 days of age. A significant decline in TCR-V frequency was observed in both the spleen (3\% of CD8+ cells) and brain (12\% of CD8+ cells) (Figure 31D). This represented nearly a 10-fold and 4-fold decline in the peak frequencies observed one month earlier (at 7 days post immunization) in the spleen and brain, respectively. Thus, this approach prolonged high-level TCR-V cell accumulation for 2 weeks post transfer. However, eventually, despite the initial robust response at the tumor site, immunization at day 7 did not induce long-term persistence of high levels of TCR-V cells.

**Immunization on day 7 post adoptive transfer of TCR-V cells enhances survival of SV11 mice**

Since day 7 immunization following adoptive transfer initially resulted in high frequency of TCR-V cells in the brain, the potential for this treatment to enhance survival of tumor-bearing SV11 mice was assessed. Cohorts of 85-day old SV11 mice (16 mice/group) received the following treatments: (i.) no treatment; (ii.) TCR-V cell adoptive transfer alone; (iii.) TCR-V cells + Tag-V only immunization on the day of transfer (day 0); or (iv.) TCR-V cells + immunization with B6/V-only Tag cells on day 7 post transfer. Mice were euthanized following the development of neurological symptoms. The presence of tumors was confirmed by gross examination. It was found that adoptive transfer of TCR-V cells + day 7 immunization significantly enhanced SV11 survival compared to mice receiving no treatment or adoptive transfer alone. The median lifespan for this cohort was 122 days (p<.0001) (Figure 32). These data suggested that the high-level accumulation of TCR-V cells in the brain resulting from day 7 immunization was responsible for delaying tumor progression.

The significant extension of median survival was similar to that observed in previous studies, whereby SV11 mice received TCR-V cell adoptive transfer combined with a prime/boost
The effect of TCR-V cell adoptive transfer followed by day 7 immunization on survival of tumor-bearing SV11 mice was assessed. Cohorts of 85-day old SV11 mice (16 mice/group) received the following treatments: (i.) no treatment; (ii.) TCR-V cell adoptive transfer alone; (iii.) TCR-V cells + Tag-V only immunization on the day of transfer (day 0); or (iv.) TCR-V cells + immunization with B6/V-only Tag cells on day 7 post transfer. Mice were euthanized following the development of neurological symptoms. The presence of tumors was confirmed by gross examination and SV11 survival in days was determined.
FIGURE 32

Immunization on Day 7 Post Adoptive Transfer of TCR-V Cells Enhances Survival of SV11 Mice

Percent survival vs Age (days)

- no treatment
- TCR-V only
- TCR-V + day 0 prime
- TCR-V + day 7 prime

median=122 days
p<.0001 vs no treatment ***/
immunization regimen (Figure 26) (Ryan and Schell, 2006). However, in those studies it was observed that when mice eventually succumbed to tumor, very few TCR-V cells could be detected in SV11 brains. When SV11 mice received immunization on day 7 alone following adoptive transfer, although high-levels were maintained for 2 weeks at the tumor site (Figure 31A), eventually TCR-V cell frequency also declined significantly, (Figure 31D). Together these data suggest that high-level persistence of cells at the tumor site is necessary for prolonged survival, and that immunization alone cannot induce long-term tumor control.

Exposure to endogenous Tag in SV11 mice compromises the ability of TCR-V cells to respond to subsequent immunization in an antigen-free environment

Immunization any later than 7 days post TCR-V cell transfer could not optimally expand TCR-V cells in SV11 mice (Figure 29). It remained possible that endogenous Tag delivered a tolerizing signal to TCR-V cells, and that persistent exposure to endogenous Tag in SV11 mice rendered TCR-V cells incapable of responding to exogenous immunization. Therefore, the ability for TCR-V cells activated by endogenous Tag to respond to subsequent activation was analyzed upon transfer to an antigen-free environment.

TCR-V cells were adoptively transferred into either unimmunized SV11 mice or B6 hosts that received immunization. TCR-V cells were re-isolated from the spleens of primary hosts 14 days post transfer, and the frequency and activation status (L-selectin) were assessed (Figure 33A). Re-isolated cells from SV11 and B6 mice were pooled separately, and $1 \times 10^5$ of either endogenous Tag-experienced (SV11 donors) TCR-V cells or B6/V-only Tag-experienced (immunized B6 donors) TCR-V cells were transferred into new B6 hosts, such that their presence would be undetectable unless expansion occurred. The same day, some recipients received B6/V-only Tag immunization. Splenocytes isolated from secondary hosts were analyzed 7 days later for frequency and effector function of TCR-V cells (Figure 33B).

The frequency and phenotype of primary isolated TCR-V cells was consistent with previous data (Figure 17) (Ryan and Schell, 2006). In unimmunized SV11 mice, TCR-V cells were incompletely activated (modest down-regulation of L-selectin) and did not accumulate (2%
Exposure to Endogenous Tag in SV11 Mice Compromises the Ability For TCR-V cells to Respond to Subsequent Immunization in an Antigen-free Environment

The ability for TCR-V cells activated by endogenous Tag in SV11 mice to respond to subsequent activation was analyzed upon transfer to an antigen-free environment. A. TCR-V cells were adoptively transferred into either unimmunized 85 day-old SV11 mice or B6 mice immunized on day 0 (6 mice/group). TCR-V cells were re-isolated from the spleens of primary hosts 14 days post transfer, and the frequency (CD8+Tetramer-V+) and activation status (CD44 and L-selectin) were assessed flow cytometrically.

B. Re-isolated cells from SV11 and B6 mice were pooled separately, and 1x10^5 of either endogenous Tag-experienced (SV11 donors) TCR-V cells or B6/V-only Tag-experienced (immunized B6 donors) TCR-V cells were transferred into new B6 hosts (6 mice/group), such that their presence would be undetectable unless expansion occurred. The same day, one-half of the recipients received B6/V-only Tag immunization (3 mice/group for each donor cell type). Splenocytes were isolated from secondary hosts 7 days later and were analyzed for TCR-V cell frequency (CD8+/Tet-V+/CD45.1+), activation (L-selectin), and effector function (production of IFNγ in response to Tag epitope-V peptide stimulation via intracellular cytokine analysis). (% = % Tet-V+/total CD8+ cells)
FIGURE 33

A. Profile of Tag immune TCR-V cells isolated from donor mice on day 14 post adoptive transfer

B. TCR-V cells re-isolated 7 days following adoptive transfer into B6 hosts
of CD8+ cells) (Figure 33A). In immunized B6 mice, TCR-V cells expanded to 14% of CD8+ cells and were fully activated, as indicated by full down-regulation of L-selectin (Figure 33A).

Upon transfer into a new B6 environment, both endogenous Tag-experienced TCR-V cells (from SV11 hosts) as well as immunization-derived Tag experienced TCR-V cells (from immunized B6 hosts) were able to expand above background (transfer to naive B6 hosts). However, the identical number (1x10^5) of donor TCR-V cells isolated from immunized B6 mice expanded to a two-fold higher frequency (6.4% of CD8+ cells) over those isolated from unimmunized SV11 hosts (3.8% of CD8+ cells) following transfer into new recipients. Additionally, a two-fold higher percentage of TCR-V cells isolated from B6 mice were functional (2.2% IFN-γ producing) compared to TCR-V cells isolated from SV11 donors (1% IFN-γ producing) (Figure 33B). These results indicate that while exposure to endogenous Tag in SV11 mice for 14 days does not render TCR-V cells incapable of responding to subsequent immunization, the response of such cells to secondary stimulation does not approach the levels achieved by TCR-V cells primed in a normal B6 environment. These data imply that endogenous Tag does not provide optimally potent stimulation towards Tag epitope-V, and suggests that exposure to persistent Tag over time could eventually render TCR-V cells tolerant in vivo.

TCR-V cells respond to immunization with wild-type Tag in B6 mice, but require Tag V-only specific primary immunization in order to respond to wild-type Tag in SV11 mice

The data from Chapter IV demonstrated that TCR-V cells respond to the immunorecessive Tag epitope V derived from the wild-type endogenous Tag in SV11 mice. Recent work has demonstrated that Tag-V is poorly cross-presented, resulting in a compromised ability to stimulate a response in the context of the immunodominant epitopes and offered a possible explanation for the subdominant nature of Tag epitope V (Otahal et al., 2005). Additionally, in B6 mice, the response to epitope V is further hampered by competition from the other dominant Tag epitope-specific T cells for access to APCs or tumor cells presenting Tag (Otahal et al., 2005). However, these precursors are centrally deleted in the thymus of Tag-tolerant SV11 mice, and should not compete with TCR-V cells. Since TCR-V cells respond to endogenous wild-type Tag in SV11 mice, the ability for these cells to respond to wild-type immunization in tumor-bearing SV11 mice was therefore questioned.
TCR-V cells were transferred into 85 day-old SV11or B6 mice. Some mice were immunized with B6/V-only Tag cells or wild-type Tag (WT-19 cells) on the day of transfer (day 0). On day 7, mice received either: (i.) B6/V-only Tag immunization, either as a primary immunization or booster immunization to B6/V-only Tag prime delivered on day 0; (ii.) WT-19 immunization, either as a prime or as a booster to B6/V-only Tag prime delivered on day 0; or (iii.) no immunization. On day 14, splenocytes and brain lymphocytes were analyzed for frequency, activation, proliferation and function (Figure 34).

Priming on day 7 with WT-19 cells was able to stimulate both an immunorecessive epitope specific response in TCR-V cells (16% of CD8+ cells) (Figure 35) as well as a dominant epitope IV specific response (9% of CD8+ cells) (data not shown) as detected in the spleens of B6 mice. However, wild-type immunization was unable to expand the TCR-V population initially activated by endogenous Tag in SV11 mice above 3% CD8+ cells in the spleen (Figure 35A). Additionally no TCR-V cells trafficked to the brain in either mouse strain (Figure 35B). This result indicated that while TCR-V cells recognize epitope V derived from the wild-type endogenous Tag in SV11 mice, epitope V specific immunization is required in SV11 mice to achieve maximal expansion with exogenous immunization.

In contrast to immunization on day 7 with wild-type Tag, immunization with V-only Tag on day 7 led to a robust response in both B6 and SV11 mice (20% and 22% tetramer+ cells/CD8+ cells, respectively) (Figure 35A). Additionally, B6/V-only Tag immunization on day 7 led to significant TCR-V cell infiltration of SV11 brains (68% of CD8+ cells) (Figure 35B). These data agree with our previous results (Figures 29 and 30) and suggests that epitope V specific immunization is necessary to achieve maximal expansion of TCR-V cells in SV11 mice, and again demonstrated that TCR-V cells are extremely responsive to epitope-V specific immunization administered 7 days following adoptive transfer.

In contrast to day 7 immunization of SV11 mice with wild-type Tag, boosting a V-only Tag specific response achieved by B6/V-only Tag immunization delivered day 0 with a wild-type Tag booster immunization delivered on day 7 led to expansion of TCR-V cells in both B6 and SV11 mice (17% and 16% tetramer+ cells/CD8+ cells, respectively) (Figure 35A). This wild-type Tag booster immunization also led to TCR-V infiltration of SV11 brains (74% of CD8+ cells) (Figure 35B). These data suggest that while epitope V specific immunization is necessary to initially expand TCR-V cells in SV11 mice, these previously activated cells can respond to a
TCR-V cells were transferred into 85 day-old SV11 or B6 mice (3 mice/group). Some mice were immunized with B6/V-only Tag cells or wild-type Tag (WT-19 cells) on the day of transfer (day 0). On day 7, mice received either: (i.) B6/V-only Tag immunization, either as a primary immunization or as a booster immunization to B6/V-only Tag prime delivered on day 0; (ii.) WT-19 immunization, either as a primary immunization or as a booster immunization to B6/V-only Tag prime delivered on day 0; or (iii.) no immunization. On day 14, TCR-V cells were isolated from the spleens and brains, and assessed by staining for Tetramer-V and CD8.
FIGURE 34

Protocol for TCR-V Cell Adoptive Transfer + Immunization With B6/V-only Tag or Wild Type Tag

EXPERIMENTAL SET-UP

<table>
<thead>
<tr>
<th>Day 0</th>
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<tbody>
<tr>
<td>TCR-V cell adoptive transfer into SV11 or B6 mice</td>
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<td>+</td>
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<tr>
<td>Primary immunization with B6/V-only Tag cells</td>
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<td>or no immunization</td>
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<th>Day 7</th>
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<tr>
<td>Booster immunization with B6/V-only Tag cells;</td>
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<td>Primary immunization with wild type Tag cells (WT-19);</td>
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<td>or no immunization</td>
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<tr>
<th>Day 14</th>
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<tbody>
<tr>
<td>Analysis</td>
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<td>TCR-V cell isolation from spleen and brain</td>
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SUMMARY OF IMMUNIZATION CONDITIONS

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells Used for Immunization on:</th>
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<tr>
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<td>Day 0</td>
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TCR-V cells were transferred into 85 day-old SV11 or B6 mice (3 mice/group). Some mice were immunized with B6/V-only Tag cells or wild-type Tag (WT-19 cells) on the day of transfer (day 0). On day 7, mice received either: (i.) B6/V-only Tag immunization, either as a primary immunization or as a booster immunization to B6/V-only Tag prime delivered on day 0; (ii.) WT-19 immunization, either as a primary immunization or as a booster immunization to B6/V-only Tag prime delivered on day 0; or (iii.) no immunization. On day 14, splenocytes (A.) and brain lymphocytes (B.) were analyzed for TCR-V cell frequency by staining for Tetramer-V and CD8. Data is presented graphically as percentage of total CD8+ cells. Error bars indicate standard deviation from the mean.
FIGURE 35

A. TCR-V cell response to prime/boost immunization with epitope V-only or wild type Tag

**SPL EN**

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<th>Prime:</th>
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<tr>
<td>Boost:</td>
<td>B6/V-only</td>
<td>WT-19</td>
<td>B6/V-only</td>
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<th>% TCR-V cells/total CD8+ lymphocytes</th>
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B. TCR-V cell response to prime/boost immunization with epitope V-only or wild type Tag

**B R A I N**

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<td>Boost:</td>
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<th>% TCR-V cells/total CD8+ lymphocytes</th>
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subsequent booster immunization with wild-type derived Tag. Assessment of SV11 mice 14 days after receiving day-0 immunization with B6/V-only Tag revealed that TCR-V cells do not expand above 6% of total CD8+ cells in the spleen (Figure 35A) and do not infiltrate the brain in significant numbers (Figure 35B). Thus, the WT-19 booster was responsible for achieving the significant TCR-V cell frequency in the spleens and brains of SV11 mice.

Finally, as previously observed (Figure 22), a prime/boost regimen with V-only Tag cells resulted in high-level TCR-V cell accumulation in the spleens of both B6 and SV11 mice at day 14 (27% and 23% tetramer+ cells/CD8+ cells, respectively) (Figure 35A). This Tag epitope V-specific immunization protocol also led to significant TCR-V cell infiltration of SV11 brains (75% of CD8+ cells) (Figure 35B). These results suggest that while epitope V specific primary immunization is necessary to initially expand TCR-V cells in SV11 mice, this primary immunization may be delivered either on day 0 and boosted on day 7, or provided on day 7 alone. In both instances, TCR-V cells expanded, achieved functional potential, and infiltrated SV11 brains.

These data clearly indicate the necessity for primary immunization specifically targeted against the immunorecessive epitope Tag V in order to elicit a maximal TCR-V cell response in SV11 mice. Once this optimal primary response has been established, it remains possible that lower thresholds are required for subsequent expansion, allowing for TCR-V cells to respond to the booster immunization with wild-type Tag. These results suggest the potential for direct stimulation of brain infiltrating TCR-V cells by the tumor to positively reinforce activation of TCR-V cells, provided they have received a peripheral signal from Tag epitope V specific immunization, licensing them to enter the brain.

Both direct and cross-presentation are required in order to achieve optimal expansion of TCR-V cells in SV11 mice following immunization with Tag V-only cells

Otahal et al. (Otahal et al., 2005) have shown that both cross- and direct-presentation are required for a maximal Tag-V specific response to be generated in B6 mice. However, in SV11 mice, TCR-V cells may be activated by tumor-derived Tag, which would presumably be cross-presented in the CLN. It was therefore asked whether cross-presentation alone was sufficient for induction of a maximal response to Tag V-only priming in SV11 mice. To
accomplish this, mice were immunized with a V-only Tag cell line that is TAP deficient (TAP-/- V-only Tag cells) - resulting in lack of surface MHC class I expression - and is thus incapable of direct presentation.

SV11 mice or B6 mice were adoptively transferred with TCR-V cells. Since TCR-V cells demonstrated maximal expansion following day 7 immunization with B6/V-only Tag cells in both SV11 and B6 mice (Figure 30), this time point was chosen for immunization following TCR-V cell adoptive transfer. Mice were immunized 7 days post transfer with either B6/V-only Tag cells (capable of direct presentation) or TAP-/- V-only Tag cells (incapable of direct presentation). Splenocytes and brain lymphocytes were assessed 7 days later for frequency (Tetramer-V+/CD45.1+) and activation status (CD44 up-regulation).

In B6 mice, TAP-/- V-only Tag cells stimulated a minimal response (5% CD8+ cells) above background (1.8% CD8+ cells) (Figure 36A), similar to previously published data (Otahal et al., 2005). However, immunization with B6/V-only Tag cells was necessary in order to achieve a maximal TCR-V cell response (27% CD8+ cells) (Figure 36A). In SV11 mice, immunization with TAP-/- V-only Tag cells led to no expansion of TCR-V cells above that observed in naive mice upon exposure to endogenous Tag (3.5%) (Figure 36B). Only following immunization with B6/V-only Tag TAP+ cells was the maximal response induced (23% of CD8+ cells in the spleen; 50% of CD8+ cells in the brain) (Figure 36B). These data suggest that the endogenous Tag delivers a suboptimal signal to TCR-V cells, similar to that delivered by TAP-/- V-only Tag cells, and that this activation is not enhanced upon immunization with a cell line that cannot directly present antigen. Additionally, these results suggest that the most critical effect of the primary immunization in SV11 mice lies in direct presentation of the immunorecessive epitope in order to achieve further expansion of TCR-V cells that may have been suboptimally activated by the endogenous Tag.

Multiple booster immunizations following adoptive transfer leads to rapid deletion of TCR-V cells and has no greater effect on SV11 survival than a single day 7 immunization

The potent effect of Tag Epitope V specific immunization on expansion of TCR-V cells was demonstrated in multiple experiments. Given the dramatic response of TCR-V cells to primary and booster immunizations, it was hypothesized that TCR-V cells could maintain control over tumor progression if they received new stimulation from exogenous immunization. In order
FIGURE 36

Both Direct and Cross-presentation Are Required in Order to Achieve Optimal Expansion of TCR-V Cells in SV11 Mice Following Immunization Targeted Against Tag Epitope-V

SV11 (9 mice) or B6 mice (9 mice) were adoptively transferred with TCR-V cells and immunized 7 days later with either B6/V-only Tag cells (capable of direct presentation) or V-only Tag TAP-/- cells (incapable of direct presentation). A separate group from each mouse strain did not receive immunization. Three mice/group were included for each experimental condition.

Splenocytes and brain lymphocytes were assessed 7 days later for frequency by Tetramer staining (Tetramer-V+/CD45.1+), activation status (CD44 up-regulation), and function (production of IFNγ in response to Tag-V peptide stimulation) of TCR-V cells in B6 mice (A.) and SV11 mice (B.).

% = % Tetramer+ cells or IFNγ producing cells/total CD8+ cells.

CD45.1 and CD44 expression was assessed on gated Tetramer+ cells.
FIGURE 36

Both direct and cross-presentation are required in order to achieve optimal expansion of TCR-V cells in SV11 mice following immunization with Tag V-only cells.
to test for this possibility, TCR-V cells were adoptively transferred into SV11 mice and followed by continuous booster immunizations at day 7, 14, 21, and 28 post transfer. The persistence of TCR-V cells in the spleen and brain was assessed by analysis of mice at 106, 113, 120 and 130 days of age following the administration of each immunization (Figure 37A). Additionally, mice were assessed for survival (Figure 37B) and were euthanized at the onset of neurological symptoms.

Multiple immunizations ultimately led to rapid deletion of TCR-V cells in both the spleen and brain by 120 days of age (Figure 37A), and did not prolong survival any longer than adoptive transfer followed by a single day 7 immunization (Figure 37B compared to Figure 32). Thus, it was concluded that TCR-V cell persistence could not be augmented by repeated immunizations despite the persistence of these cells for at least 3 weeks following day 7 immunization (Figure 31D).

**Pre-conditioning with anti-CD40 mAb results in rapid accumulation of functional TCR-V cells in SV11 spleens and brains**

The ability to activate naive T-CD8 cells *in vivo* using agonistic anti-CD40 mAb (α-CD40) has been reported in the literature (Bennett et al., 1998; Diehl et al., 1999; Mackey et al., 1998a; Maxwell et al., 1999; Nakajima et al., 1998; Schoenberger et al., 1998; Toes et al., 1998a), and may involve several mechanisms, including maturation of antigen presenting cells (Caux et al., 1994; Cella et al., 1996; Grewal and Flavell, 1998; Koch et al., 1996; Mackey et al., 1998b; Moodycliffe et al., 1999; Terheyden et al., 2000) (Figure 38A) or direct stimulation of the T cell (Bourgeois et al., 2002). Staveley-O'Carroll et al. (Staveley-O'Carroll et al., 2003) have shown that α-CD40 pre-conditioning enhances Tag epitope I specific T cell (TCR-I) responses in the osteosarcoma 501 mouse model. Since immunorecessive Tag epitope V specific TCR-V cells recognize endogenous Tag in naive SV11 mice [(Ryan and Schell, 2006) and Figure 17], the effect of agonistic α-CD40 conditioning *in vivo* - in the absence of immunization - on the activation of TCR-V cells against the endogenous Tag and accumulation at the tumor site was determined.

The endogenous Tag triggers a measurable, albeit weak response by TCR-V cells, but does not result in accumulation of T cells in the brain [(Ryan and Schell, 2006) and Figures 20
A. TCR-V cells were adoptively transferred into SV11 mice, followed by immunization with B6/V-only Tag cells (5x10^7 cells/dose) on days 7, 14, 21, and 28 post transfer (3 mice/group). The persistence of TCR-V cells in the spleens and brains was assessed by Tetramer-V analysis at 105, 113, 120 and 130 days of age following the administration of 1, 2, 3, or 4 immunizations, respectively.

B. Another group of SV11 mice (14 mice) were transferred with TCR-V cells, immunized on days 7, 14, 21, and 28 post transfer, and assessed for survival. Mice were euthanized at the onset of neurological symptoms.
FIGURE 37

A. TCR-V cells are rapidly deleted in SV11 mice following multiple boosters

![Bar chart showing % TCR-V cells in spleen and brain over age in days with immunizations 1X, 2X, 3X, 4X]

B. Multiple boosters has no greater effect on SV11 lifespan than single immunization on day 7 post transfer

![Graph showing percent survival over age in days with TCR-V cells + 4X boost median lifespan = 128 days and no treatment median lifespan = 105 days]
A. Agonistic anti-CD40 monoclonal antibody (α-CD40) can be administered in vivo to activate immature professional antigen presenting cells (APCs). Agonistic α-CD40 may substitute for CD4+ T cell help by taking the place of CD40L (CD154) and engaging CD40 on APC. The interaction of CD40/CD154 stimulates maturation of the APC, resulting in up-regulation of activation and co-stimulatory molecules. A fully mature APC may now induce successful activation of naïve T-CD8 cells specific for self or tumor antigens.

B. TCR-V cells were adoptively transferred into 85 day-old SV11 or B6 mice. Some mice were administered αCD40 agonistic mAb (or control IgG Ab) via the intraperitoneal (i.p.) route on the day before (day -1) and after (day +1) transfer. Groups of mice were assessed at day 7 and day 14 post transfer to determine frequency and activation of TCR-V cells in the spleen and brain by staining for CD8, Tetramer-V and CD44, and for function by IFN\(\gamma\) production in response to Tag-V specific peptide stimulation via intracellular cytokine assay.
An anti-tumor response against endogenous self-antigen can be induced in vivo with $\alpha$-CD40 mAb

A.
FIGURE 38

B.

General protocol for adoptive transfer of naïve TCR-V cells and sensitization to endogenous Tag

Day 0
adoptive transfer

TCR-V

Day -1 and Day +1
α-CD40 mAb

SV11 85 days old

Day 7
ex vivo analysis

Isolate TCR-V cells from spleen and brain
A possible explanation for the meager response against the endogenous Tag could be attributed to peripheral tolerance induced by the APC (Garza et al., 2000; Heath and Carbone, 2001b; Kusmartsev et al., 2005; Miller et al., 1998; Probst et al., 2005; Shortman and Heath, 2001; Sotomayor et al., 2001; Steinman, 2003; Steinman et al., 2000). It has been shown that such a tolerizing signal can be overcome by the administration of agonistic α-CD40, bypassing the need for CD4+ T cell help, and thereby activating APCs that present tumor-derived Tag (Diehl et al., 1999; Diehl et al., 2000; Sotomayor et al., 1999) (Figure 38A). Therefore, the potential for the limited response of TCR-V cells toward the endogenous Tag to be enhanced in vivo by administration of α-CD40 was investigated.

TCR-V cells were adoptively transferred into 85 day-old SV11 or B6 mice. Some mice were administered α-CD40 agonistic mAb (or control IgG Ab) via the intraperitoneal (i.p.) route on the day before and after transfer (Figure 38B). Groups of mice were assessed at day 7 and day 14 post transfer to determine frequency and activation of TCR-V cells in the spleen and brain by staining for CD8, Tetramer-V and CD44, and for function by IFNγ production in response to Tag-V specific peptide stimulation. TCR-V adoptive transfer plus control IgG mAb alone led to minor expansion of cells (2.5% of CD8+ cells) in the spleen compared to B6 mice (Figure 39A). These cells were activated, as indicated by up-regulation of CD44 and acquisition of IFNγ production, but less than 1% of CD8+ cells were detectable in the brain (Figure 39A). By comparison, administration of α-CD40 induced a dramatic TCR-V cell response against the endogenous Tag in both the spleens (33% of total CD8+ cells) and brains (42% of CD8+ cells) of SV11 mice by day 7 post transfer (Figure 39A). This robust expansion represented a 20-fold higher TCR-V cell accumulation compared to the response of TCR-V cells in naive SV11 mice in the absence of CD40 activation. This expanded population homogeneously expressed CD44, and a proportion of these cells were functional as indicated by 18% of CD8+ splenocytes and 16% of CD8+ brain lymphocytes that produced Tag-V specific IFNγ (Figure 39A).

Importantly, not only did agonistic α-CD40 administration lead to accumulation of high TCR-V cell frequencies in SV11 spleens (34-45% of total CD8+ cells) this treatment also promoted early entry of TCR-V cells into the brain, representing 30-47% of total CD8+ cells at the tumor site by one week post transfer. Previously, accumulation of TCR-V cells in the brain was not observed until day 14, following a prime/boost immunization (Figures 20 and 22), or following day 7 immunization (Figure 30). Thus, for a rapidly progressing tumor, this 7-day
window in achieving accumulation of tumor specific T cells at the tumor site has the potential to drastically alter therapeutic efficiency.

The dramatic early effect of pre-conditioning with α-CD40 mAb is not maintained

Surprisingly, despite the dramatic initial response of TCR-V cells to α-CD40 administration, TCR-V cell frequency declined to 10% of CD8+ cells in the spleen and 11% of CD8+ cells in the brain by 14 days post transfer (Figure 39B). Approximately half of these cells produced IFNγ in the spleen and brain, respectively at this time point (Figure 39B). These results indicate that while α-CD40 treatment pre-sensitized TCR-V cells to activation against endogenous Tag, and induced significant accumulation of TCR-V cells both in the periphery and brain, this effect was short-lived, since high levels of functional TCR-V cells were not maintained.

A combination of pre-conditioning against endogenous Tag and well-timed exogenous immunization synergistically maintains TCR-V frequency at the tumor site

The data indicate that α-CD40 administration and optimally-timed immunization provided differential kinetics of high frequency TCR-V infiltration of SV11 tumors following adoptive transfer. Agonistic α-CD40 mAb induced a robust early response in the spleen and brain that peaked at 7 days and subsequently declined. Immunization at day 7 dramatically expanded TCR-V cells in both organs by day 14 post transfer. However, neither immunotherapeutic modality resulted in long-term persistence of TCR-V cells at the tumor site when delivered in isolation. Given the heavy tumor burden that already exists at 85 days, accumulation of TCR-V cells at the tumor site 14 days post transfer may be too late to exert a substantial anti-tumor effect. Thus, the potential for a combination of these individual treatments to cooperatively induce both rapid accumulation and maintenance of brain-infiltrating TCR-V cells in tumor-bearing SV11 mice was determined.

TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Mice were administered α-CD40 agonistic mAb the day before and after transfer. On day 7 post transfer, mice were immunized with Tag-V only cells. Mice were assessed on day 14 post transfer in
A. Pre-conditioning against endogenous Tag with $\alpha$CD40 results in rapid accumulation of functional TCR-V cells in SV11 spleens and brains

TCR-V cells were adoptively transferred into 85 day-old SV11 or B6 mice. Some mice were administered $\alpha$CD40 agonistic mAb (or control IgG Ab) via the intraperitoneal (i.p.) route on the day before (day -1) and after (day +1) transfer. Groups of mice from each treatment condition (3 mice/group) were assessed on day 7 post transfer to determine frequency and activation of TCR-V cells in the spleen and brain by staining for CD8, Tetramer-V and CD44, and function by IFN\(\gamma\) production in response to Tag-V specific peptide stimulation via intracellular cytokine assay.

B. The dramatic early effect of pre-conditioning with $\alpha$CD40 mAb is not maintained

Groups of SV11 and B6 mice receiving the same treatments as in Part A (3 mice/group) were assessed on day 14 post transfer to determine frequency of TCR-V cells in the spleen and brain by staining for CD8 and Tetramer-V, and function by IFN\(\gamma\) production in response to Tag-V specific peptide stimulation via intracellular cytokine assay. A representative example of flow cytometric data for one of the mice that received combinatorial therapy is pictured.

C. A combination of pre-conditioning against endogenous Tag and well-timed exogenous immunization synergistically maintains TCR-V frequency at the tumor site

TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Mice were administered $\alpha$CD40 agonistic mAb the day before and after transfer. Mice also received immunization with B6/V-only Tag cells 7 days post transfer (combinatorial treatment). Mice were analyzed at 105 days of age (20 days following combinatorial treatment) to assess the frequency (staining for CD8 and Tetramer-V) and function (IFN\(\gamma\) production in response to Tag-V specific peptide stimulation via intracellular cytokine assay) of TCR-V cells that persisted in the spleen and brain. A representative example of flow cytometric data for one of the mice that received combinatorial therapy is pictured.
FIGURE 39

A.

High levels of activated and functional TCR-V cells accumulate in SV11 spleens and brains following pre-conditioning with α-CD40

(TCR-V cells isolated at day 7 post transfer + α-CD40)
FIGURE 39

B. TCR-V cell frequency is not maintained in SV11 spleens and brains following pre-conditioning with α-CD40

C. TCR-V frequency is maintained in SV11 mice following α-CD40 pre-conditioning combined with day 7 immunization
order to determine TCR-V frequency and function in the spleen and brain. The results show that immunization administered 7 days post TCR-V cell transfer + α-CD40 rescued the dramatic decline in cell frequency observed on day 14 following TCR-V cells + α-CD40 alone. Tetramer-V+ cells represented 21% and 52% of CD8+ cells in the spleen and brain, respectively (Figure 39C). A proportion of these cells were functional, indicated by 12% and 35% IFNγ producing cells detected in the spleen and brain, respectively (Figure 39C). These results demonstrate that timely immunization at day 7 post-transfer is capable of sustaining the dramatic accumulation of TCR-V cells at the tumor site achieved by α-CD40 pre-conditioning.

Combinatorial immunotherapy promotes long-term TCR-V cell persistence at the tumor site and enhances survival of SV11 mice

Given the ability for α-CD40 and day 7 immunization to sustain early TCR-V cell accumulation in the brains of SV11 mice for 2 weeks, it was important to establish how long TCR-V cells persisted following combinatorial therapy. Thus, the longevity of TCR-V cells at the tumor site following immunotherapy was assessed. To this end, SV11 mice were analyzed at 40 days post adoptive transfer – the same time point at which it was observed that the population of TCR-V cells expanded by day 7 immunization alone had waned significantly (Figure 31D). TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Mice were administered one of three treatments (6 mice/group): (i.) α-CD40 agonistic mAb the day before and after transfer; (ii.) immunization with B6/V-only Tag cells 7 days post transfer; or (iii.) both α-CD40 agonistic mAb the day before and after transfer and immunization with B6/V-only Tag cells 7 days post transfer (combinatorial immunotherapy). Mice were analyzed at 125 days of age (40 days following initiation of immunotherapy) in order to assess the frequency of TCR-V cells that persisted in the spleen and brain.

In SV11 mice that had received combinatorial immunotherapy, high levels of TCR-V cells persisted at the tumor site (Figure 40A). This result appeared to be more than an additive effect, since the percentage of TCR-V cells in the brain at this late time point (mean of 47% Tet-V+ cells/total CD8+ cells) was drastically greater than that observed at the identical time point following either treatment administered alone (mean of 11% Tet-V+/total CD8+ cells for day 7 immunization; mean of 5% Tet-V+/total CD8+ cells for αCD40 stimulation) (Figure 40A). Thus, the ability for combinatorial therapy to sustain high frequency of TCR-V cells at the tumor site is
FIGURE 40

Combinatorial Immunotherapy Promotes Long-Term TCR-V Cell Persistence at the Tumor Site and Enhances Survival of SV11 Mice

A. The longevity of TCR-V cells at the tumor site following immunotherapy was assessed. TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Mice were administered one of three treatments (6 mice/group): (i.) αCD40 agonistic mAb the day before and after transfer; (ii.) immunization with B6/V-only Tag cells 7 days post transfer; or (iii.) both αCD40 agonistic mAb the day before and after transfer and immunization with B6/V-only Tag cells 7 days post transfer (combinatorial immunotherapy). Mice were analyzed at 125 days of age (40 days following initiation of immunotherapy) to assess the frequency of TCR-V cells that persisted in the spleen and brain. Data for all of the mice in the study is presented in graphical form. Error bars indicate standard error of the mean.

B. The effect of immunotherapeutic regimens based on pre-conditioning with αCD40 mAb on the survival of tumor-bearing SV11 mice was assessed. Cohorts of 85-day old SV11 mice (16 mice/group) received the following treatments: (i.) αCD40 mAb administration alone without TCR-V cell adoptive transfer; (ii.) αCD40 + TCR-V cell adoptive transfer; or (iii.) αCD40 + TCR-V cell adoptive transfer + day 7 immunization with B6/V-only Tag cells (combinatorial immunotherapy). Mice were euthanized following the development of neurological symptoms. The presence of tumors was confirmed by gross examination and SV11 survival in days was determined.
FIGURE 40

Combinatorial Immunotherapy Synergistically Promotes Long-Term TCR-V Cell Persistence at the Tumor Site and Enhances Survival of SV11 Mice

A. Frequency of TCR-V cells in SV11 mice at 125 days of age

B. Percent survival vs. Age (days)
likely due to a synergistic mechanism.

It was also important to determine whether the early tumor infiltration of functional TCR-V cells induced by \( \alpha \)-CD40 treatment corresponded to control of disease progression in SV11 mice. The effect of immunotherapeutic regimens based on pre-conditioning with \( \alpha \)-CD40 mAb on the survival of tumor-bearing SV11 mice was assessed. Cohorts of 85-day old SV11 mice (16 mice/group) received the following treatments: (i.) \( \alpha \)-CD40 mAb administration alone without TCR-V cell adoptive transfer; (ii.) \( \alpha \)-CD40 + TCR-V cell adoptive transfer; or (iii.) \( \alpha \)-CD40 + TCR-V cell adoptive transfer + day 7 immunization with B6/V-only Tag cells (combinatorial immunotherapy). Mice were euthanized following the development of neurological symptoms, the presence of tumors was confirmed by gross examination, and SV11 survival in days was determined.

TCR-V adoptive transfer coupled with \( \alpha \)-CD40 did significantly increase SV11 survival to a median age of 125 days \((p<.0001)\) (Figure 40B). This result was dependent upon its effect on the transferred cells, since administration of \( \alpha \)-CD40 alone had no greater therapeutic value than no treatment or TCR-V cell adoptive transfer only (Figure 40B and Figure 26, respectively). These findings demonstrate that pre-conditioning against endogenous Tag with \( \alpha \)-CD40 promoted SV11 survival, implying that early accumulation of TCR-V cells in the brain is critical for tumor control. However, eventually, all mice in this treatment group succumbed to tumor burden, consistent with the lack of TCR-V cell persistence following \( \alpha \)-CD40 + TCR-V cell adoptive transfer (Figure 39B and Figure 40A).

Finally, the potential for combinatorial immunotherapy to enhance survival of tumor-bearing SV11 mice was assessed. The results demonstrate that TCR-V cell adoptive transfer coupled with \( \alpha \)-CD40 and day 7 immunization enhanced SV11 survival even further, to a median lifespan of 144 days (Figure 40B), which was statistically significant compared to either treatment modality delivered separately (\( p \) value = .0012 vs TCR-V + \( \alpha \)-CD40 (Figure 40B); \( P < .0001 \) vs. TCR-V + day 7 immunization (Figure 32)). Thus, the combination of \( \alpha \)-CD40 pre-conditioning + day 7 immunization promoted not only long-term persistence of brain-infiltrating TCR-V cells, but significantly extended SV11 survival, suggesting that maintenance of high levels of functional TCR-V cells at the tumor site is essential for long-term tumor control.
In summary, individual treatments of optimally-timed exogenous Tag-V immunization or pre-conditioning against endogenous Tag with α-CD40 mAb both promoted high-level accumulation of TCR-V cells in the brains of SV11 mice with significant tumors (Figure 30 and Figure 39). This observation correlated with comparable enhancement of survival (Figure 32 and 40B). However, neither immunotherapeutic modality resulted in long-term persistence of TCR-V cells at the tumor site when delivered in isolation (Figure 31D, Figure 39B, and Figure 40A), and neither had a significantly greater advantage over the other in the ability to control tumor progression (Figure 32 and 40B). In contrast, therapy consisting of α-CD40 mAb combined with day 7 immunization led to long-term persistence of TCR-V cells in the brain, correlative with dramatic delay of disease progression (Figure 40A and B). These data are summarized in Table 2.

The size of SV11 tumors at the time of initiation of TCR-V cell mediated combinatorial immunotherapy may predict therapeutic effect

In all of the previous investigations, the effect of immunotherapy on control of SV11 tumor progression was only determined by the duration of SV11 survival and tumor size at the time of analysis. Thus, while combinatorial immunotherapy significantly prolonged SV11 survival, the actual effect of TCR-V cell mediated therapy on tumor growth was not ascertained. The only method by which the effect of combinatorial therapy could be correlated with tumor progression was to monitor tumor size in vivo. Thus, a longitudinal study was conducted in which SV11 tumors were assessed by magnetic resonance imaging (MRI) at three time points following administration of combinatorial therapy. Prior to initiation of therapy, 85 day-old SV11 mice were examined by MRI scan in order to determine the baseline tumor size. The following day, combinatorial therapy was initiated, and mice received α-CD40 mAb (on days -1 and +1) + TCR-V cell adoptive transfer + day 7 immunization. Mice were subsequently imaged by MRI scan at 105 day of age (20 days post therapy) and 125 days of age (40 days post therapy) to assess tumor volume. Following the final scan, SV11 mice were euthanized, tumors were isolated and prepared for histological analysis.

A control group included 4 SV11 mice that did not receive any treatment. These mice were assessed at 85 days and if they survived long enough - at 105 days of age. None of these
85 day old SV11 mice received the following treatments: (i.) none; (ii.) TCR-V cell adoptive transfer only; (iii.) TCR-V cell adoptive transfer + immunization with B6/V-only Tag cells on day 0; (iv.) TCR-V cell adoptive transfer + immunization with B6/V-only Tag cells on day 7; (v.) TCR-V cell adoptive transfer + αCD40 mAb; (vi.) TCR-V cell adoptive transfer + αCD40 mAb + immunization with B6/V-only Tag cells on day 7. At 7, 14, or 40 days post transfer, lymphocytes recovered from spleens and brains of representative mice were analyzed for the presence of CD8⁺/Tet-V⁺ cells. Relative TCR-V cell frequencies in the spleen and brain are represented as: -, +, ++, ++++, or ++++, according to their frequency calculated as percentage of total CD8⁺ cells. Data shown is the mean of 3 separate experiments, which included 3 mice/group.

Relative TCR-V cell frequency (as % total CD8⁺ cells)
- none detectable
+ 0 - 5% CD8⁺ cells
++ 6 - 20% CD8⁺ cells
+++ 21-40% CD8⁺ cells
++++ > 40% CD8⁺ cells

Additional groups of mice (14/group) were assessed for survival following each treatment. Data shows the median lifespan for each treatment group. Statistical significance was determined by single-factor ANOVA, and validated using log-rank test. Values of p<0.05 were considered significant.
Table 2.

Synergistic Maintenance of TCR-V Cells in SV11 Mice Following Combinatorial Immunotherapy

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 7 Spleen</th>
<th>Day 7 Brain</th>
<th>Day 14 Spleen</th>
<th>Day 14 Brain</th>
<th>Day 40 Brain</th>
<th>Median survival (days)</th>
<th>p value (vs. NT)</th>
</tr>
</thead>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>101</td>
<td>NA</td>
</tr>
<tr>
<td>TCR-V only</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>104</td>
<td>.1022</td>
</tr>
<tr>
<td>TCR-V + day 0 prime</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>105</td>
<td>.0063**</td>
</tr>
<tr>
<td>TCR-V + day 7 prime</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>122</td>
<td>&lt;.0001***</td>
</tr>
<tr>
<td>TCR-V + α-CD40</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>125</td>
<td>&lt;.0001***</td>
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<tr>
<td>TCR-V + α-CD40 + day 7 prime</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>144</td>
<td>&lt;.0001***</td>
</tr>
</tbody>
</table>

TCR-V + α-CD40 + day 7 immunization significance
(.0012** vs. TCR-V + α-CD40; 0001*** vs. TCR-V + day 7 prime)
mice survived to the final time point and all had succumbed to tumor by 109 days of age. A representative example of an untreated mouse is shown in Figure 41A. Tumor volume at 85 days of age was 6 mm$^3$, which progressed significantly by 105 days of age to 255 mm$^3$ (tumors indicated with red arrow). Tumor progression was associated with extreme hydrocephalus due to obstruction of ventricle outflow by the invading tumor, as well as blood-brain-barrier breakdown, reflected by leakage of the gadolinium contrast agent into the CSF (yellow arrows) (Figure 41A). Histological analysis of this tumor indicated that the large tumor mass had completely filled the ventricle (Figure 42B). An example of normal choroid plexus tissue from a B6 mouse is shown for comparison (Figure 42A).

In SV11 mice that received combinatorial therapy, two distinct trends were observed. When tumors were of substantial size at the time of therapy initiation (between 60 mm$^3$ – 80 mm$^3$), very little control over tumor progression was observed and tumors grew progressively, reaching a final volume of 280 mm$^3$ – 310 mm$^3$ (Figure 43). MRI analysis of a representative mouse (#2734) is depicted at 85 days of age (72 mm$^3$) and 125 days of age (284 mm$^3$) (Figure 41B). Upon histological analysis, these tumors were extremely dense, had obliterated the ventricles, and were characterized by areas of necrosis and accumulations of erythrocytes, indicating high-vascularity and hemorrhage within the tumor, even following brain perfusion (Figures 42C and D). Additionally, these mice exhibited severe correlative neurological symptoms, including hydrocephalus and ataxia.

In contrast, when tumor volume was relatively small at the onset of therapy (between 4 mm$^3$ - 12 mm$^3$), tumors progressed at a significantly slower pace, reaching final volumes of 28 mm$^3$ – 77 mm$^3$ (Figure 43). MRI analysis of a representative mouse (#2746) is depicted at 85 days of age (4 mm$^3$) and 125 days of age (28 mm$^3$) (Figure 41C). Histologically, these tumors varied in size and morphology, but were clearly less advanced than in mice whose tumors had progressed significantly. In some mice, areas of normal choroid plexus tissue were found nearby zones of tumor growth (Figure 42E). In most of these tumors, the tumor cells were more diffuse, with pockets of ventricle space still evident, and necrosis and hemorrhage were rare (Figure 42F). As these mice appeared neurologically stable, the size and histology of these tumors correlated with significantly less advanced disease. Tumor progression in treated SV11 mice with small tumors at 85 days (#2732 and #2746) and large tumors at 85 days (#2727...
A longitudinal study was conducted in which SV11 tumors were assessed by gadolinium-enhanced T1-weighted MRI analysis at three time points following administration of combinatorial therapy. Prior to initiation of therapy, 85 day-old SV11 mice were imaged in order to determine the baseline tumor size. The following day, combinatorial therapy was initiated, and mice received αCD40 mAb (on days -1 and +1) + TCR-V cell adoptive transfer + day 7 immunization. Mice were subsequently analyzed by MRI scan at 105 day of age (20 days post therapy) and 125 days of age (40 days post therapy) in order to assess tumor volume. Control SV11 mice that did not receive treatment were assessed at 85 days and 105 days of age (when applicable).

A. Untreated SV11 mouse (#2754) imaged at 85 days and 105 days of age.
B. SV11 mouse with large tumor burden at onset of combinatorial therapy (#2734) imaged at 85 days and 125 days of age.
C. SV11 mouse with small tumor burden at onset of combinatorial therapy (#2746) imaged at 85 days and 125 days of age.

Red arrows indicate tumor. Yellow arrows indicate leakage of gadolinium contrast agent into ventricles.
FIGURE 41

In Vivo Visualization of Tumor Progression in SV11 Mice Following Combinatorial Immunotherapy by Magnetic Resonance Imaging

SV11 # 2754
no treatment
85 days of age
Tumor volume: 6 mm$^3$

A

105 days of age
Tumor volume: 255 mm$^3$

SV11 # 2734
combinatorial therapy
85 days of age
Tumor volume: 72 mm$^3$

B

125 days of age
Tumor volume: 284 mm$^3$

SV11 # 2746
combinatorial therapy
85 days of age
Tumor volume: 4 mm$^3$

C

125 days of age
Tumor volume: 28 mm$^3$
At the conclusion of the longitudinal MRI study (Figure 41) in which SV11 tumors were monitored for tumor progression by MRI analysis following initiation of combinatorial therapy (αCD40 + TCR-V cell adoptive transfer + day 7 immunization), mice (3 mice/group) were euthanized, tumors were isolated, fixed in formalin, and prepared for histological analysis via hemotoxylin and eosin stain. All treated mice were 125 days of age (40 days post therapy).

A. Normal choroid plexus tissue from B6 mouse (100X magnification)

B. SV11 tumor from untreated mouse (#2754) (105 days of age;100X magnification)

C. Tumor from an SV11 mouse (#2734) with large tumor at initiation of combinatorial therapy (200X magnification)

D. Tumor from an SV11 mouse (#2727) with large tumor at initiation of combinatorial therapy (40X magnification)

E. Tumor from an SV11 mouse (#2732) with small tumor at initiation of combinatorial therapy (100X magnification)

F. Tumor from an SV11 mouse with small tumor (#2746) at initiation of combinatorial therapy (200X magnification)
FIGURE 42

SV11 Tumor Burden at Initiation of TCR-V Cell Mediated Combinatorial Immunotherapy May Predict Tumor Progression
A longitudinal study was conducted in which SV11 tumors were assessed by
gadolinium-enhanced T1-weighted MRI analysis at three time points following
administration of combinatorial therapy. Prior to initiation of therapy, 85 day-old SV11
mice were scanned in order to determine the baseline tumor size. The following day,
combinatorial therapy was initiated, and mice received αCD40 mAb (on days -1 and +1)
+ TCR-V cell adoptive transfer + day 7 immunization (SV11 #2727, 2734, 2746, 2732).
Mice were subsequently analyzed by MRI scan at 105 day of age (20 days post
therapy) and 125 days of age (40 days post therapy) in order to assess tumor volume.
Control SV11 mice (SV11 #2754) that did not receive treatment were assessed at 85
days and 105 days of age (when applicable).
Data is presented as tumor volume (mm$^3$) at each analysis time point.
FIGURE 43

SV11 Tumor Burden at Initiation of TCR-V Cell Mediated Combinatorial Immunotherapy May Predict Tumor Progression
and #2734) is shown in comparison with an SV11 mouse with a small tumor at 85 days that did not receive treatment (#2754) (Figure 43).

These data clearly indicate that disease status at the time of treatment initiation limits the effect of TCR-V cell mediated immunotherapy toward SV11 tumors. Although similar frequencies of TCR-V cells accumulate in the brains of SV11 mice, the functionality of these cells in their ability to control tumor progression is significantly different. In no instance did TCR-V obliterate or even reduce tumor size. Rather, for small tumors, TCR-V cells appeared to hold tumor progression in check, suggesting that new tumor cell growth was balanced by destruction of established tumor cells. When tumors were large at the onset of therapy, TCR-V cells exerted some anti-tumor control, as treated SV11 mice survived significantly longer than untreated mice. However, tumor burden obviously outweighed the therapeutic capacity of infiltrating TCR-V cells, since tumors continued to grow, progressing to 5 times their initial volume at the start of therapy, despite persistent accumulation of TCR-V cells within the tumor stroma.

**CONCLUSIONS**

These results demonstrate that the response of TCR-V cells in SV11 mice may be enhanced by activation against both endogenous and exogenous forms of Tag. The potentially tolerizing effect posed by persistent endogenous Tag on TCR-V cells in the tumor host environment can be subverted by pre-conditioning SV11 mice with α-CD40 mAb. TCR-V cells are also extremely sensitive to exogenous Tag provided by cellular immunization. However, in order to achieve maximal expansion with immunization, TCR-V cells must be activated specifically against immunorecessive Tag epitope V that is available in a directly presented form. Optimal TCR-V cell response to immunization is also influenced by a critical window of time following adoptive transfer, within which dramatic expansion is possible, and is negatively affected by excessively frequent immunizations. While modulations of TCR-V cell activation against both endogenous or exogenous immunization are achievable with α-CD40 mAb or immunization, respectively, leading to dramatically augmented *in vivo* responses, both therapeutic strategies face limitations, namely lack of T cell persistence. However, the combination of the two modalities, which potentially target unique aspects of optimizing TCR-V
cell responses, demonstrated dramatic synergistic effects, both in TCR-V cell persistence and SV11 survival. It can also be concluded that an optimized T cell response does not always predict clinical efficacy. Tumor burden at the onset of combinatorial immunotherapy correlated with the capacity for TCR-V cells to control disease progression, suggesting that a threshold of tumor size exists for which TCR-V cells may exert a therapeutic effect, and beyond which the tumor overwhelms immunotherapeutic potential.

**DISCUSSION**

Adoptive immunotherapeutic approaches for the treatment of cancer are often coupled with strategies aimed at optimizing the T cell response in vivo in order to subvert tolerance and maintain functional T cell persistence. The studies presented here attempted to explore approaches targeted at augmenting the in vivo response of TCR-V cells toward the immunorecessive Tag epitope-V in order to enhance the anti-tumor effect of TCR-V cells following adoptive transfer into SV11 mice. The results demonstrate the identification of multiple strategies with the potential to significantly improve a weak tumor antigen-directed response. As these individual approaches target separate immunotherapeutic pathways, the capacity for combined modalities to synergistically transform incomplete activation into robust TCR-V cell accumulation and anti-tumor function was observed.

**α-CD40 Conditioning**

In the SV11 model, adoptively transferred TCR-V cells are activated by endogenous antigen in the CLN, but fail to accumulate or demonstrate therapeutic efficacy. Thus, it is highly likely that APCs in the tumor-draining node are insufficiently mature for proper induction of an anti-tumor response. The sub-optimal response of TCR-V cells to endogenous Tag reflects observations made in other experimental models, in which tumor-specific T-CD8 cells proliferated in tumor draining lymph nodes at all times during tumor progression, indicating that tumor antigen was continuously available for cross-presentation to T cells in proximal lymph nodes. However, the resulting T cell population was unable to exert anti-tumor effects (Robinson et al., 1999).
Weak activation by self or tumor antigens may be due to inefficient cross-presentation or insufficient DC maturation necessary for cross-priming. Evidence for the latter is supported by the observation that in vivo administration of agonistic α-CD40 mAb can overcome this shortcoming and promote robust expansion of tumor specific T-CD8 cells (Hernandez et al., 2001; Kurts et al., 1997; Kurts et al., 1999; Liu et al., 2002b; Ohlen et al., 2002; van Mierlo et al., 2002). Thus, the potential for in vivo conditioning with CD40-targeted mAbs to alter the maturation status of APCs - thereby amplifying weak T-CD8 cell mediated anti-tumor responses - is now well-appreciated. The APC-targeted licensing effect of CD40 ligation may act in cooperation with other immunotherapeutic approaches, as it was observed to be essential for tumor rejection upon immunization with antigen-loaded dendritic cells (Mackey et al., 1998a; Mackey et al., 1998b). Moreover, α-CD40 conditioning has been shown to overcome the tolerogenic effect of optimal-length peptide administration, giving rise to a great interest in manipulating this pathway in the field of cancer vaccines (Diehl et al., 1999).

The effect of α-CD40 conditioning on augmentation of tumor-targeted immune responses has been demonstrated elegantly in vivo. Using a syngeneic mouse tumor model expressing an antigen derived from the early region 1A of human adenovirus type 5 (E1A), van Mierlo et al. showed that α-CD40 mAb stimulation led to induction of T-CD8 cell mediated eradication of established CD40-negative tumors via a mechanism that required CD40 expression on host cells (van Mierlo et al., 2002). It was later demonstrated, that the inadequate nature of the anti-tumor T cell response was not due to direct Ag presentation by the tumor cells, but from presentation of tumor-derived Ag by non-activated CD11c+ APCs. Although cross-presentation resulted in division of naive T-CD8 cells in tumor draining lymph nodes, a productive immune response was not established. Treatment of tumor-bearing mice with agonistic α-CD40 mAb resulted in systemic efflux of T-CD8 cells with robust effector function capable of eradicating established tumors. Efficacy of α-CD40 treatment required CD40 ligation of host APCs, as adoptive transfer of CD40-proficient tumor-specific TCR transgenic T-CD8 cells into CD40-deficient tumor-bearing mice did not lead to productive anti-tumor immunity following in vivo CD40 triggering. It was concluded from these studies that depending on their activation state, DCs orchestrate the outcome of T-CD8 cell-mediated immunity against tumors, leading either to an ineffective immune response or potent anti-tumor immunity (van Mierlo et al., 2004).
Many of the studies aimed at exploring the conditioning effect of \( \alpha \)-CD40 mAb treatment were performed in models that assessed T-CD8 responses to transplantable tumors, in which the tumor antigen was a foreign antigen. However, tolerance induction is likely to be more stringent in DCs presenting self/tumor-derived antigens, potentially posing an elevated threshold for eliciting immune responses against tumors that express self antigens. One of the intriguing findings from the current investigations concerned the enhanced priming of TCR-V cells against the endogenous Tag in vivo via administration of agonistic \( \alpha \)-CD40 mAb. This activation promoted dramatic expansion of TCR-V cells and early infiltration of the brain. Agonistic \( \alpha \)-CD40 mAb combined with TCR-V adoptive transfer was also capable of exerting control over tumor progression. A similar effect was demonstrated in transgenic mice that express an LCMV tumor antigen as a self antigen in the pancreas, but were not transgenic for SV40 Tag, and therefore did not develop tumors. Only peptide + \( \alpha \)-CD40 mAb could induce a potent T-CD8 cell response that rapidly induced diabetes (Garza et al., 2000).

Success for \( \alpha \)-CD40 pre-conditioning in the induction of T-CD8 immunity towards spontaneous tumors has been previously reported. Nguyen et al. demonstrated in transgenic mice that develop SV40 Tag induced pancreatic insulinomas expressing a model self antigen from LCMV, that tumor antigens were cross-presented by bone marrow-derived APCs. This resulted in activation of T-CD8 cells specific for immunodominant viral antigen epitopes that were insufficient for promotion of tumor rejection. However, treatment with agonistic \( \alpha \)-CD40 mAb enhanced APC activation and inhibition of tumor growth (Nguyen et al., 2002). In another SV40 Tag-induced mouse model of osteosarcoma, \textit{in vivo} CD40 ligation overcame tolerance of adoptively transferred TCR Tg T-CD8 cells specific for the dominant Tag epitope I, resulting in T cell persistence, activation, and establishment of memory (Staveley-O'Carroll et al., 2003). However, the ability for \( \alpha \)-CD40 conditioning to enhance activation and anti-tumor effectiveness of T-CD8 cells specific for immunorecessive epitopes in spontaneous models had not previously been addressed.

Interestingly, the dramatic expansion of TCR-V cells in SV11 mice was short-lived, and TCR-V cells appeared to undergo deletion, as only low frequencies were detectable in the spleen or brain 14 days later. A similar situation was observed in a transplantable mouse melanoma model, whereby administration of agonistic \( \alpha \)-CD40 mAb accelerated deletion of tumor antigen specific T-CD8 cells (Kedl et al., 2001). In another report, \( \alpha \)-CD40 mAb pre-conditioning rescued T-CD8 cells specific for human adenovirus type 5 E1A from tolerance.
following vaccination with a minimal epitope. However, despite effective T-CD8 priming with the combination of systemic α-CD40 mAb and peptide vaccine, the T-CD8 cell response quickly waned and failed to protect mice against tumor challenge, an effect attributed to systemic persistence of the peptide (den Boer et al., 2001; Diehl et al., 1999). Finally, α-CD40 mAb administration was observed to mediate T cell deletion in an autoimmunity model (Mauri et al., 2000). These reports indicate that the ultimate deletional effect of α-CD40 mAb is not limited to our experimental protocol.

The results presented here contrast with other reports in the literature, including the finding that in vivo conditioning with α-CD40 mAb enhanced survival of activated T-CD8 cells by preventing deletion following superantigen administration (Maxwell et al., 1999). An explanation for the discrepancy between observations of rapid T cell deletion following α-CD40 mAb (including ours) and this study may relate to a correlation between persistent antigen expression and increased susceptibility to α-CD40 mAb-mediated deletion. Another study reported that in vivo administration of α-CD40 mAb induced bystander proliferation of non-antigen specific memory T-CD8 cells as well as division of antigen-specific cells, which required expression of CD40 on APCs and IL-15 (Koschella et al., 2004). Thus, α-CD40 mAb mediated re-stimulation of host memory T-CD8 cells in SV11 mice might explain the co-accumulation of non-Tag specific endogenous T-CD8 cells with TCR-V cells in SV11 tumors. Given the limited supply of cytokine sinks, it remains possible that TCR-V cells pre-conditioned with α-CD40 are not maintained because of competition for survival with non-Tag specific T cells.

Some of the first investigations of the effect of α-CD40 mAb conditioning on treatment of solid tumors reported that CD40 expression was required on host APCs but not necessarily by the tumor, and that CD40-mediated immunotherapy was most efficient in mice bearing heavy tumor loads, suggesting that a critical quantity of tumor-derived antigen was necessary for APC-mediated priming of T-CD8 responses (French et al., 1999; Todryk et al., 2001). Tumor antigens shed from tumors or apoptotic tumor cells taken up by APCs are a likely source of tumor antigens that drive T-CD8 cell mediated responses (Albert et al., 1998b) and high antigen dose appears to be important for this process (Kurts et al., 1998c). Due to its capacity to induce maturation in DCs, the dramatic effect of in vivo CD40 triggering on the expansion of T-CD8 in the enhancement of tumor immunity has predominately been attributed to the enhanced capacity of DCs to prime a T cell response by substitution for CD4+ T cell help. However, one
publication did report that expression of CD40 on CD8+ T cells was critical for the receipt of CD4+ T cell help directly through CD40, and that this interaction was fundamental for the generation of CD8+ T cell memory population (Bourgeois et al., 2002). In the SV11 mouse system, CD40 is constitutively expressed on CD11c+ DCs both in the CLN and tumor site (data not shown), strongly implicating these APCs as the targets of α-CD40 conditioning against Tag in SV11 mice.

These findings offer promise for the treatment of solid tumors, since such malignancies may be of substantial size at the point when symptoms arise. As shown in other solid tumor models, effective α-CD40 mAb-mediated therapy is likely a race between having sufficient tumor antigen present for priming of T cells and SV11 mice becoming overwhelmed by disease. These data indicate that a limited therapeutic window exists for optimal effectiveness of immunotherapy. Thus, approaches that expand this window by slowing tumor progression while retaining enough antigen for proper T cell activation may yield enhanced therapeutic efficacy.

**Immunization**

Levels of tolerance may depend on the tumor type and manner in which immunity against the tumor was induced, and T cells stimulated by quiescent DCs may regain responsiveness following removal from the presence of persistent antigen (Aichele et al., 1995; Redmond et al., 2003). Additionally, tolerance may be reversible with optimally designed immunotherapeutic manipulations. It has been demonstrated that residual low avidity T-CD8 cells specific for a tumor self-antigen in the pancreas responded to vaccination, resulting in rejection of tumor challenge without induction of autoimmunity (Morgan et al., 1998). In another model, peptide boosting of memory cells primed by virus vaccination prevented the deletion of cytotoxic IFNγ producing memory cells specific for the tumor, but these cells could not control tumor progression. However, functional capacity was regained, indicated by the ability of epitope specific cells to reject tumor challenge upon removal of T cells to an antigen-free environment (den Boer et al., 2004). The ability of such T-CD8 cells to exhibit cytolytic activity towards tumor cells and differentiate into responsive memory cells suggests that under the proper conditions, tumor-reactive cells can be rescued from tolerance, and harnessed in the periphery for anti-tumor reactivity.

Similarly, the studies presented here show that initial exposure of TCR-V cells to endogenous Tag, while weakly activating, does not completely tolerize TCR-V cells, as they
may still respond to Tag-V stimulation 2 weeks after transfer upon isolation and re-transfer into an antigen-free environment (B6 hosts). However, by this point these cells responded less potently to stimulation than TCR-V cells activated in B6 primary hosts, suggesting that a tolerizing signal had already been initiated. These studies also demonstrate that immunization specifically against epitope V was necessary for eliciting full activation and intra-tumoral accumulation – an effect that may be attributed to the poor efficiency of Tag-V cross-presentation (Otahal et al., 2005) – and required direct presentation of Tag-V by the cell line used for immunization. In order for TCR-V cells to accumulate in SV11 mice at levels comparable to those observed in B6 mice, immunization with TAP-competent B6/V-only Tag cells was necessary. Provided that Tag-V specific immunization had initiated expansion, TCR-V cells were capable of responding to a booster immunization with wild-type Tag cells. These data suggested that TCR-V cells that had been primed with B6/V-only cells in the periphery retained the potential to respond to epitope V directly presented by tumor cells expressing wild-type Tag in the brain – potentially explaining the high-level accumulation of BrdU positive TCR-V cells in the tumor.

It has recently been shown that antigen presentation by immature DCs resulted in meager activation and proliferation of T-CD8 cells that did not down-regulate CCR7 or L-selectin. These central memory-like T cells were not deleted, but responded to recall stimulation in secondary hosts to yield a fully activated T cell population harboring effector function (Dumortier et al., 2005). A similar situation may possibly explain the need for secondary immunization on day 7 in SV11 mice. TCR-V cells exposed to endogenous Tag post transfer are neither ignorant nor tolerized – rather these cells fully retain the capacity to develop robust T cell responses following receipt of the proper signal.

**Timing of immunization**

An intriguing finding from Chapter IV concerned the significantly weaker TCR-V cell response to exogenous Tag-V targeted immunization of SV11 mice compared to that observed in B6 mice when immunization was delivered on the day of adoptive transfer. These studies also sought to identify the basis for this observation. It was hypothesized that due to immediate activation by the endogenous Tag, TCR-V cells might be refractory to immunization administered coincident with adoptive transfer. To our knowledge, the effect of endogenously expressed antigen on the response of adoptively transferred T cells to subsequent immunization
delivered at different time points post transfer has not previously been explored. These investigations found that TCR-V cells are critically sensitive to time of immunization delivery, and suggest that these cells are eventually tolerized by persistent endogenous Tag, as has been observed in other models of persistent antigen expression (Hernandez et al., 2002; Hernandez et al., 2001; Redmond et al., 2003).

Neither B6/V-only Tag priming administered prior to or after day 7 was as effective as immunization at 7 days post-adoptive transfer. A possible explanation for this result may relate to the previous finding that TCR-V cells peak between day 4 and day 7 post transfer in unimmunized SV11 mice in response to endogenous Tag. It can be proposed that TCR-V cells engaged by endogenous Tag in the CLN are unable to simultaneously respond to antigen derived from exogenous immunization. Unpublished observations from Dr. Pavel Otahal in our laboratory indicate that the antigen derived from B6/V-only cells is cleared within 24 hours post i.p. injection. Coupled with the knowledge of the instability of Tag-V/MHC complexes, immunization-derived Tag-V may be in limited supply at the point when TCR-V cells have recovered from primary (endogenous) activation and regain responsiveness to secondary (exogenous) immunization. Therefore, it is highly likely that the peak levels of TCR-V cells generated by the endogenous Tag, combined with the time allowed for re-distribution of TCR-V cells to lymphoid organs, allows for a maximal response to antigen derived from B6/V-only Tag cellular immunization that is limited by a 24-hour window in which to stimulate an in vivo response when immunization is administered on day 7. Optimal timing of immunization correlated with trafficking of TCR-V cells to the brain, accumulation at the tumor site, and significant increase in survival, resulting in a large proportion of actively proliferating TCR-V cells at 3 weeks post-immunization. Together, these data indicate that when a tumor expresses self-antigen, activation of adoptively transferred immunorecessive tumor epitope-specific cells may be highly sensitive to timing of exogenous immunization.

**Frequency of Immunization**

An important parameter affecting the response to vaccination and resulting immune response is the frequency of immunization. In response to DC vaccination of cancer patients administered 4x over 6 weeks, while the frequency of antigen-specific T cells increases, this effect is short-lived as detected in peripheral blood (Banchereau et al., 2001a; Palucka et al., 2003). Such low blood levels of T cells could result from migration to the tumor, or from overly
frequent vaccination, as multiple doses of immunization induce deletional tolerance (Aichele et al., 1995; Dubois et al., 1998; Masopust et al., 2001). In line with the latter possibility, the current studies showed that repeated immunizations of SV11 mice actually led to faster deletion of TCR-V cells, illustrating that “more” is not necessarily “better.” Mouse and human studies of vaccination against infectious agents (Kaech et al., 2002; Zinkernagel, 2003) indicate that for an optimal response, priming should be followed by a booster 4-6 weeks later. However, these rules may not apply to treatment of chronic disease such as cancer. The cancer setting can be compared to chronic viral infection, in which T cells become exhausted from chronic Ag presentation (Wherry et al., 2003a; Zajac et al., 1998), and it is possible that reactivation of tumor specific T-CD8 cells through vaccination requires carefully-designed scheduling of timing and frequency.

In respect to limitations posed in overly enthusiastic administration of immunotherapy in a tumor model, intra-tumoral immunotherapy of Her-2/neu mice with IL-12 and GM-CSF induced transient tumor regression. However, repeated immune therapy resulted in loss of tumor-specific T-CD8 cells, increase in Tregs, and progressive loss of anti-tumor efficacy associated with chronic immune therapy, despite enhanced intra-tumoral IFNγ production (Nair et al., 2006). Such findings have important clinical implications for cancer immunotherapy, in that induction of anti-tumor immunity may rapidly be counteracted by regulation, and repeated stimulation may result in progressive loss of therapeutic efficacy due to increased suppressor activity and eventual immune exhaustion. The TCR-V cell-mediated response to SV11 tumors demonstrated similar loss of efficacy following repeated stimulations, whereby TCR-V cells were rapidly deleted and tumors progressed at a rate comparable to tumors in mice receiving single immunization doses. Thus, standard vaccination protocols may have a limited window of efficacy in the established disease setting, strongly implicating the need to examine kinetics and frequency of immunization in addition to choosing the optimal tumor antigen-expressing vector for targeting the unique tumor type.

**Combination Immunotherapy**

Following day 7 immunization, the fraction of proliferating TCR-V cells in the brain ultimately declined, and TCR-V cell numbers fell accordingly. Thus, while optimally timed immunization had some effect on TCR-V cell recruitment and SV11 survival, high TCR-V cell levels failed to persist long-term and mice eventually succumbed to tumor. Additionally, despite
the initial expansion and early intra-tumoral accumulation of TCR-V cells activated by α-CD40 conditioning, this response was extremely short-lived. In contrast to individual treatments delivered alone (pre-sensitization with agonistic α-CD40 mAb or exogenous immunization), a combination of α-CD40 mAb prior to transfer plus day 7 immunization promoted maintenance of high TCR-V cell levels in SV11 mice – both in the spleen and brain – and had the most dramatic effect on survival. In comparison to the rapid disappearance of TCR-V cells following α-CD40 alone, and ultimate decline of cells after day 7 immunization alone, combinatorial therapy promoted high-level TCR-V cell persistence long-term in the brain, even at the time of death.

Given the myriad avenues of immune escape from tumor-targeted immunotherapy, the need for protocols designed to include multiple anti-tumor modalities in combinatorial immunotherapeutic regimens has become increasingly evident. Strategies for the enhancement of adoptive immunotherapy may include combinations of cytostatic drugs, IL-2 administration, tumor-targeted immunization, pre-injection of TNFα at the site of DC vaccination, hematopoietic stem cell transfer (HSC), and irradiation (Banchereau and Palucka, 2005; Cameron et al., 1990; Ganss et al., 2002; Lake and Robinson, 2005; Overwijk et al., 2003; Pardoll and Allison, 2004).

An example of a tripartite immunotherapy approach in the laboratory was demonstrated by the 3-armed strategy used to target aggressive transplanted lymphomas in mice. To provide for increased antigen expression by DCs in vivo, HSCs were transduced with genes encoding tumor antigen, and transplanted into irradiated mice. This combined the effect of antigen delivery to DC progenitors and the benefits associated with autologous bone marrow transplantation (BMT). In order to bypass tolerance induction, BMT was followed with infusion of mature lymphocytes (DLI). Finally, DCs were expanded in vivo with Flt3 ligand and activated with α-CD40 mAb (Cui et al., 2003). The combination of BMT using transduced HSCs, systemic agents that generate and activate DCs, and mature T-cell infusion led to significant expansion and activation of tumor antigen-specific T cells and provided potent antigen-specific immunotherapy for an aggressive established tumor. In the clinic, combination immunotherapy of myeloma patients has shown that high-dose chemotherapy, autologous HSC transplantation, and adoptive transfer of in vivo primed and ex vivo re-stimulated T cells, followed by booster immunizations counteracted the induced lymphopenia and fostered memory T cell responses (Rapoport et al., 2005). These studies demonstrate the potential for successful laboratory studies to translate into effective therapeutic clinical regimens.
The success of combinatorial therapy lies in the potential for the separate modalities to complement one another, thereby inducing synergistic augmentation of the anti-tumor response. The present studies indicate that α-CD40 conditioning and properly-timed immunization do enhance the TCR-V cell response to SV11 tumors synergistically. One explanation for this observation is that the two modalities target different forms of tumor antigen – whereby α-CD40 mAb conditions the response towards the endogenous Tag and immunization provides a potent source of exogenous antigen. Additionally, while α-CD40 conditioning is likely to target APCs presenting tumor-derived Tag-V, cellular immunization may affect the APCs that acquire the antigen for cross-presentation as well as provide the TCR-V cells with a form of directly-presented Tag-V. Given the requirements for both cross- and direct-presentation in the maximal expansion of TCR-V cells, it may be surmised that α-CD40 conditioning optimizes the initial response to endogenous Tag following adoptive transfer, generating a potently activated TCR-V cell population that responds avidly to “secondary” encounter with antigen derived from immunization delivered on day 7.

Another possible explanation is that the T cells gain enhanced capacity to survive, as would be indicated by up-regulation of apoptosis-resistance genes such as bcl-2, or receptors for survival cytokines such as IL-7 or IL-15 (Fry et al., 2001; Li et al., 2001b; Liu et al., 2002a; Schluns et al., 2000; Weng et al., 2002). The elicitation of third signal pro-inflammatory cytokines, including IL-12 (Parmiani et al., 2000; Schmidt and Mescher, 1999), TNFγ (Gorelik and Flavell, 2001; Pape et al., 1997a; Pape et al., 1997b), or IL-23 (Oppmann et al., 2000) may relate to the separate contributions of either α-CD40 mAb or the immunization to the observed effect (Hernandez et al., 2002). Differential levels and kinetics of cytokine expression induced separately by α-CD40 conditioning and immunization may also contribute to the integrated effects of combinatorial therapy. Another candidate for the observed persistence of TCR-V cells is expression of the Ca²⁺- independent protein kinase Cθ (PKCθ) T cell activation-involved enzyme. While it does not appear to be required for antigen-induced T cell proliferation, PKCθ is important for T cell survival and complete differentiation into cytokine-producing T-CD8 cells (Barouch-Bentov et al., 2005).

Alternatively, the effect of combinatorial therapy may concern an issue of T cell numbers. Administration of α-CD40 mAb induces high-levels of TCR-V cells in the periphery that can be detected 7 days following adoptive transfer. Thus, the precursor frequency of TCR-
V cells with the potential to respond to immunization delivered on day 7 is substantially larger than when TCR-V cells are transferred without α-CD40 mAb. The quality of TCR-V cells activated by mature APCs that have experienced α-CD40 mAb conditioning may also differ dramatically from that of TCR-V cells primed by potentially-tolerizing APCs. Immunization on day 7 may act as a secondary immunization to a population of TCR-V cells optimally primed by fully-mature APCs presenting endogenous self antigen.

The effects of combinatorial therapy in the SV11 model reflect observations made in other reports, including the afore-mentioned study published by Kedl et al. in which in vivo conditioning with agonistic α-CD40 mAb in melanoma-bearing mice accelerated the deletion of tumor-antigen-specific T cells (Kedl et al., 2001). However, long-term survival and function of tumor-antigen-specific T cells could be achieved when viral immunization with tumor antigen and α-CD40 mAb treatment were combined. These results similarly demonstrate the capacity for immunization to rescue the limitations associated with administration of α-CD40 individually, namely deletion of adoptively transferred T-CD8 cells. In another model, Bronte et al. (Bronte et al., 2003) demonstrated that systemic administration of agonistic α-CD40 mAb enhanced the therapeutic potential of genetic vaccination against an endogenous murine leukemia virus to induce tumor-specific T-CD8 cells capable of rejecting tumor challenge. In this model, the adjuvant effect of α-CD40 mAb was maximal when delivered at the time of tumor challenge compared to time of immunization, implicating antigen derived from the growing tumor as contributory to enhanced elicitation of α-CD40 mAb-driven, T-CD8 cell-mediated anti-tumor effect. Finally, in a renal cell adenocarcinoma mouse model, synergy was observed in the T-CD8 cell mediated anti-tumor response elicited following α-CD40 mAb combined with IL-2 administration (Murphy et al., 2003). It was postulated that while α-CD40 mAb may function to bypass the need for CD4+ T cell help by direct APC maturation, the contribution of IL-2 may relate to enhanced survival of expanded T cells or to direct regulation of DCs (Granucci et al., 2002). Thus in the SV11 model, immunization may provide the IL-2 and other cytokines necessary for optimizing priming and survival of TCR-V cells.

A possible explanation for the dramatic effect of combinatorial therapy on SV11 survival concerns the early infiltration of TCR-V cells into the brain following α-CD40 conditioning. When mice are adoptively transferred with TCR-V cells at 85 days of age, tumors are already of significant size. Administration of α-CD40 mAb drives TCR-V cells into the tumor site by 7 days
post transfer (92 days of age). Optimally timed immunization (delivered at day 7) promotes trafficking of TCR-V cells into the brain between 4 and 7 days after immunization and also promotes high-level accumulation of T cells in tumors. However, at this point the tumors have had another week to grow. Given the progressive nature of SV11 tumors, this 7-day window may prove to be critical for achieving high-levels of T cells within the tumor in the battle between tumor growth and T cell anti-tumor effector function.

**Tumor burden**

The results also indicate that tumor size at the onset of therapy may determine the ultimate effectiveness of this form of immunotherapy, whereby smaller tumors correlated with enhanced tumor control. The finding that tumors were never eradicated suggests that a delicate balancing act exists between the tumor and the T cells, whereby T cells hold tumors in equilibrium, preventing further progression. Similar observations have been reported following the adoptive transfer of TCR-Tg T cells specific for model tumor antigen at different times during tumor progression, allowing for observation of the kinetic battle between the growing tumor and nascent anti-tumor response. In one study, influenza nucleoprotein (NP) specific T cells proliferated, acquired cytotoxic activity and infiltrated the tumor, irrespective of tumor size, but rejected tumors only when at a favorable lymphocyte to tumor ratio (Cordaro et al., 2000). Hanson et al. (Hanson et al., 2000) similarly demonstrated that adoptively transferred T-CD8 cells rejected established tumors depending on dose and time of transplant. In the presence of large tumors, tumor-specific T cells failed to reject the tumor, but retained functionality and were capable of rejecting small tumors.

On the other hand, tumors must be large enough to provide enough antigen for cross-presentation. Support for this requirement was shown in a transplantable tumor model and adoptive transfer of TCR-Tg cells for influenza nucleoprotein (NP) (Cordaro et al., 2000). Small tumors could not induce a rejection response, although they were not ignored. T cells were activated, and infiltrated the tumor bed, but exerted no effect. However, when T cells were transferred into mice bearing large tumor masses, the tumor was rejected, correlating with earlier infiltration of high T cell levels into the tumor. It was determined that efficacy of adoptive transfer is dictated by the size of the tumor mass at the time of transfer, predicting that treatment of minimal residual disease with adoptive cellular therapy will fail unless vaccination is provided (Cordaro et al., 2000). Similarly, Ohashi and colleagues found that in RIP-Tag2 mice
expressing the LCMV glycoprotein as a tumor antigen, adoptive immunotherapy was enhanced in mice with large tumor burdens, suggesting that increased antigen promoted responsiveness of T cells for tumor control (Nguyen et al., 2002). For SV11 mice, it appears that sufficient antigen is available for priming of adoptively transferred TCR-V cells when immunotherapy is initiated at 85 days of age. However, despite the augmented effect of combinatorial treatment, all mice eventually succumbed to tumor, suggesting that SV11 tumors are simply too large for T cell therapy to win the kinetic battle. Alternatively, changes may occur in the T cells or at the tumor site, permitting the tumor to circumvent immune surveillance.

These studies reveal the complexities involved in designing immunotherapeutic approaches to cancer. It was shown that mode of tumor antigen presentation, timing of therapy initiation, kinetics and frequency of immunization, and selection of in vivo adjuvants must be carefully chosen in order to elicit optimal anti-tumor effectiveness. These investigations illustrate the powerful potential for combinatorial therapy to synergistically and successfully augment the capacity for immunorecessive tumor epitope-specific T-CD8 cells to target solid tumors.
CHAPTER VI

TUMOR ESCAPE IS ASSOCIATED WITH CD8+ T CELL ANERGY AT LATE TIME POINTS, BUT CAN BE PREVENTED BY MULTIPLE TREATMENTS WITH COMBINATORIAL IMMUNOTHERAPY

GOALS

The results presented thus far indicate that despite an initial dramatic response, long-term TCR-V cell persistence, and significant delay of disease in tumor-bearing SV11 mice, combinatorial immunotherapy targeted against an immunorecessive tumor epitope ultimately fails to eliminate tumors. It was hypothesized that the TCR-V cells are eventually rendered anergic, thereby unable to maintain effective control over tumor progression. Additionally, it was proposed that the SV11 tumors adopt mechanisms to escape TCR-V cell surveillance, either by avoiding recognition or by direct inhibition. In order to investigate potential mechanisms that contribute to these observations, this question was addressed from two perspectives.

The T cell: It is possible that the failure of TCR-V cells to control tumor progression beyond that observed in SV11 mice that had received combinatorial therapy is due to intrinsic limitations in the T cells. To address some of these possibilities, TCR-V cells that accumulated in the brain following combinatorial therapy were assayed for non-responsiveness.

The Tumor: Alternatively, while TCR-V cells may retain anti-tumor function, the tumor/tumor microenvironment may have been altered such that TCR-V cells are prevented from exerting continued control over tumor progression.

OVERVIEW

The following experiments sought to investigate mechanisms that contribute to the ultimate loss of tumor control despite long-term maintenance of TCR-V cells in both the periphery and brains of SV11 mice post combinatorial immunotherapy. The results indicate that several factors contribute to the ability of SV11 tumors to escape TCR-V cell surveillance, including anergic rendering of TCR-V cells, tumor infiltration by bone marrow-derived DCs, expression of inhibitory receptors and ligands by TCR-V cells and DCs, and lack of TCR-V cell memory establishment. However, in spite of the failure of TCR-V cells from the initial transfer to exert continued anti-tumor function, SV11 tumors remained receptive to a second round of
combinatorial therapy that included a fresh adoptive transfer of naïve TCR-V cells, implicating the anergic T cells in the promotion of tumor escape.

**INTRODUCTION**

In contrast to the ability to elicit an initially dramatic anti-tumor T cell response, prolonged control of tumor progression presents many challenges. It may generally be assumed that large tumor burdens would be more difficult to eradicate by tumor-specific T cell responses than small tumors (Schreiber, 1993). However, it has been shown that peripheral tissue antigens must be presented at relatively high levels to be processed via the cross-presentation pathway to naïve CD8 T cells (Kurts et al., 1998c). It is possible that small tumors are ignored, simply because antigen presentation levels remain below a critical threshold. Alternatively, despite recognition and activation of tumor specific T cells, the elicited response may remain ineffective.

Three major categories of immune escape exist for tumors under immunological surveillance: (i.) changes in the T cell (deletion and anergic rendering); (ii.) changes in the tumor (loss of antigen or MHC class I expression); and (iii.) changes in the tumor microenvironment (infiltration of suppressor cell populations and production of modulatory cytokines). Tumor-reactive T cells may be rendered non-functional due to induction of anergy (Deeths et al., 1999; Gajewski et al., 2006; Lyman et al., 2005; Staveley-O'Carroll et al., 1998) or exhaustion (Gallimore et al., 1998b; Moskophidis et al., 1993). Lack of co-stimulatory molecules on tumor cell surfaces may result in partial activation of T cells and lead to their deletion or inability to generate a full-blown effector T cell response (Chen et al., 1993). Additionally, growth of the tumor could result from generation of escape variants, leading to loss of MHC class I expression (Garrido et al., 1997; Seliger et al., 1997) and production of immunosuppressive cytokines such as TGFβ by tumor cells may inactivate tumor specific T cells (Ranges et al., 1987). Expression of Fas ligand on tumor cells can lead to apoptosis of tumor-reactive T cells (Hahne et al., 1996; O'Connell et al., 1996; Strand et al., 1996; Walker et al., 1997) and defective infiltration of the tumor stroma may account for failure of the immune system to mediate tumor rejection, despite effective generation of effector T-CD8 cells (Singh et al., 1992; Wick et al., 1997). Suppressor cell types such as T regulatory cells and tolerizing APCs may infiltrate the tumor stroma to
interact with other cells or produce cytokines that dampen the immune response. Expression of inhibitory receptors on T cells following activation occurs naturally as a mode of controlling an overly robust systemic immune reaction. However, in the context of persistent antigen, perpetual expression of inhibitory receptors on tumor-reactive T cells and their cognate ligands by other cell types that infiltrate the tumor may function to turn the T cells off. This leads to lack of correlation between accumulation of tumor-infiltrating T cells and anti-tumor control.

Multiple cell types, located either endogenously in the tumor environment or recruited during the course of the immune response, may express inhibitory receptors or induce T cells to express inhibitory markers, ultimately dampening the anti-tumor response. The engagement of such inhibitory receptors delivers a negative signal to the tumor-specific T cell, which may alter downstream signaling, activation, maturation, proliferation, effector function, and persistence (Zou, 2005). Potential interactions include the binding of CTLA-4 or PD-1 expressed on tumor-infiltrating T cells to cognate B7 family ligands expressed on tumors or tumor-associated APCs, and recent studies have documented the significant contribution of the PD-1/B7-H1 pathway to the inhibition of anti-tumor responses (Blank et al., 2004; Curiel et al., 2003; He et al., 2004; Hirano et al., 2005; Iwai et al., 2002; Strome et al., 2003).

The expression of PD-1 is naturally induced on activated T-CD8 cells in order to modulate immune responses following clearance of antigen, and plays a critical role in preventing autoimmunity. During chronic antigen expression, the receptor remains up-regulated and through engagement of its ligands – B7-H1 and B7-DC – initiates downstream signaling cascades that may induce tolerance or non-responsiveness in T cells. Inhibition manifests through the prevention of T cell proliferation and effector functions, and through the induction of T cell apoptosis. This inhibitory pathway has been implicated in limiting T-CD8 cell responses to cancer – which may be viewed as a form of chronic antigen - and expression of B7-H1 and B7-DC is detectable on multiple human and murine tumor types, coincident with PD-1 expression on non-responsive T cells specific for tumor antigens (Dong et al., 2002; Hirano et al., 2005; Iwai et al., 2002). Evidence for the key contribution of PD-1 to permitting immune escape by tumors has been described in reports that document the capacity for blockade of PD-1/PD-1L interactions to restore T-CD8 cell responsiveness in vitro and in vivo (Blank et al., 2004; Curiel et al., 2003; He et al., 2004; Hirano et al., 2005; Iwai et al., 2002; Strome et al., 2003).
Generally speaking, multiple escape mechanisms synergize to counteract an elicited anti-tumor response. In the separate mouse models of SV40 Tag-induced tumors, various different means of immune escape have been demonstrated. SV11 mice respond dramatically to the adoptive transfer of T-CD8 cells specific for Tag epitope IV, whereby tumors regress and T cells persist (Schell et al., 1999; Schell and Tevethia, 2001). In the 501 osteosarcoma model, peripheral tolerance through deletion of Tag-specific T-CD8 cells is observed (Schell et al., 2000; Staveley-O’Carroll et al., 2003). In the RIP-Tag4 model, T-CD8 cells specific for Tag epitope I are rapidly deleted in the periphery and tumors develop resistance to Tag-IV cell mediated therapy (Otahal et al., 2006). Given the numerous possible avenues of tolerance that might contribute to immune escape by SV11 tumors in spite of an initially dramatic TCR-V cell response, several potential mechanisms were investigated. These analyses included (i.) assessment of TCR-V cell anergy induction, (ii.) alterations in cell surface expression patterns of SV11 tumors, and (iii.) changes in the cellular composition within the tumor microenvironment.
RESULTS

At late time points post combinatorial treatment, SV11 tumors remain sensitive to TCR-V cell mediated immunotherapy

To investigate the possibility that SV11 tumors develop resistance to TCR-V-mediated immunotherapy, a second round of combinatorial therapy (fresh transfer of TCR-V cells + \( \alpha \)-CD40 mAb + day 7 immunization) was delivered at 125 days of age – 40 days following the original adoptive transfer. TCR-V cells from the second transfer expressed both CD45.1 and CD45.2, enabling these cells to be distinguished from TCR-V cells from the first transfer (marked with CD45.1 only). Control groups included SV11 mice that received the initial round of TCR-V mediated immunotherapy + either (i.) a second dose of \( \alpha \)-CD40 mAb only at 125 days of age; (ii.) a second dose of \( \alpha \)-CD40 mAb + day 7 immunization at 125 days of age; (iii.) a dose of TCR-IV cells (that recognize the immunodominant Tag epitope IV) combined with \( \alpha \)-CD40 + Tag-IV specific immunization at 125 days of age; or (iv.) no treatment. Mice were monitored for morbidity and survival (Figure 44).

A second round of TCR-V cell mediated adoptive immunotherapy (TCR-V cells + \( \alpha \)-CD40 triggering + day 7 immunization) resulted in a dramatic increase in SV11 survival (181 day median) (Figure 44A). Control groups demonstrated an effect similar to that achieved with a single round of TCR-V cell mediated immunotherapy (Figure 40) (142 day median lifespan for \( \alpha \)-CD40 mAb at second dose and 147 day median lifespan for \( \alpha \)-CD40 + immunization at second dose) (Figure 44A). The significantly enhanced survival of mice that were administered the complete second round of therapy correlated with tumor-site accumulation of TCR-V cell populations from both transfers. In representative mice analyzed at 3 different time points, TCR-V cells from the second transfer (CD45.1+CD45.2+) peaked in the spleen by 160 days of age (70% of total TCR-V cells) and peaked in the brain by 190 days of age (35% of total TCR-V cells). However, at all time points TCR-V cells from the initial bolus (CD45.1 only) persisted at high frequencies in both the spleen and brain (Figure 44B). These data demonstrate that while TCR-V cells are not deleted following combinatorial immunotherapy, a second bolus of naïve TCR-V cells was necessary in order to maintain an anti-tumor effect. These results indicate that SV11 tumors remain sensitive to TCR-V cell mediated immunotherapy at 40 days post initiation of combinatrial treatment. Had the second adoptive transfer of TCR-V cells shown no effect,
FIGURE 44

At Late Time Points Post Combinatorial Treatment, SV11 Tumors Remain Sensitive to TCR-V Cell Mediated Immunotherapy

A. At 85 days of age, SV11 mice were administered combinatorial therapy (TCR-V cells expressing CD45.1, α-CD40 agonistic mAb conditioning + day 7 immunization). A second round of combinatorial therapy (fresh transfer of CD45.1/CD45.2 TCR-V cells + α-CD40 mAb + day 7 immunization) was delivered at 125 days of age – 40 days following the original adoptive transfer. TCR-V cells from the second transfer were marked with both CD45.1/CD45.2, enabling these cells to be distinguished from TCR-V cells from the first transfer (marked with CD45.1 only).

Control groups included SV11 mice that received the initial round of TCR-V mediated immunotherapy + either (i.) a second dose of α-CD40 mAb only at 125 days of age; (ii.) a second dose of α-CD40 mAb + day 7 immunization at 125 days of age; (iii.) a dose of TCR-IV cells (that recognize the immunodominant Tag epitope IV) combined with CD40 + Tag-IV specific immunization at 125 days of age; or (iv.) no treatment. Mice were monitored for morbidity and survival. Ten mice were included in each group.

B. Tumors from representative mice at age 140 days, 160 days, and 190 days were analyzed for frequency of tumor-infiltrating TCR-V cells assessed by staining for Tetramer-V, CD8, CD45.1 and CD45.2.
FIGURE 44
SV11 Tumors Remain Sensitive to TCR-V Cell Mediated Combinatorial Adoptive Immunotherapy

A.

<table>
<thead>
<tr>
<th>Day 85 treatment</th>
<th>Day 125 treatment</th>
<th>median survival (days)</th>
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</thead>
<tbody>
<tr>
<td>no treatment</td>
<td>none</td>
<td>105</td>
</tr>
<tr>
<td>TCR-V/αCD40/immunization</td>
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<td>142</td>
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<tr>
<td>TCR-V/αCD40/immunization</td>
<td>αCD40/immunization</td>
<td>147</td>
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<tr>
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<td>TCR-V/αCD40/immunization</td>
<td>181</td>
</tr>
<tr>
<td>TCR-V/αCD40/immunization</td>
<td>TCR-V/αCD40/immunization</td>
<td>184</td>
</tr>
</tbody>
</table>

median survival: 184 days
p<.0001*** vs. αCD40 or αCD40/immunization at second dose
p=.3098 vs TCR-V cells at second transfer

median survival: 181 days
p<.0001*** vs. αCD40 or αCD40/immunization at second dose
FIGURE 44

SV11 Tumors Remain Sensitive to TCR-V Cell Mediated Combinatorial Adoptive Immunotherapy

B.

<table>
<thead>
<tr>
<th></th>
<th>SPLEEN</th>
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<th>BRAIN</th>
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<tbody>
<tr>
<td></td>
<td>transfer</td>
<td>transfer</td>
<td>transfer</td>
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</tr>
<tr>
<td>CD8</td>
<td>15%</td>
<td>91% 9%</td>
<td>38%</td>
<td>93% 7%</td>
</tr>
<tr>
<td>CD45.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>17%</td>
<td>30% 70%</td>
<td>27%</td>
<td>85% 15%</td>
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<tr>
<td>CD45.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>9%</td>
<td>75% 25%</td>
<td>36%</td>
<td>65% 35%</td>
</tr>
<tr>
<td>Tetramer-V</td>
<td>CD45.1</td>
<td>CD45.2</td>
<td>Tetramer-V</td>
<td>CD45.2</td>
</tr>
</tbody>
</table>

140 DAYS
160 DAYS
190 DAYS
this might suggest that escape variants had arisen that ceased to express the Tag-V epitope. However, these data suggest that the tumor had not intrinsically changed, as it remained susceptible to TCR-V mediated therapy. Importantly, despite persistence of TCR-V cells from the initial round of therapy, immunization + α-CD40 could not promote anti-tumor activity in these remaining cells, suggesting an intrinsic alteration in the persisting T cells, rendering them non-responsive.

The effect of a secondary transfer with TCR-V cells was compared to the effect of T-CD8 cells specific for Tag-IV - an epitope already known to mediate tumor regression. Had SV11 tumors had not been susceptible to TCR-V mediated therapy (due to loss of the Tag-V epitope), it remained possible that they retained expression of other Tag epitopes, and could therefore respond to other forms of Tag mediated immunotherapy. Thus as an additional control, the possibility for SV11 tumors to respond to immunodominant Tag-IV epitope mediated therapy was investigated. SV11 mice that had received an original round of combinatorial immunotherapy (TCR-V cells + α-CD40 + immunization) at 85 days of age, received a bolus transfer of TCR-IV cells (that recognize the immunodominant Tag epitope IV) combined with α-CD40 + Tag-IV specific immunization at 125 days of age. It was found that this treatment also prolonged SV11 survival (184 day median) (Figure 44A), which correlated with approximately equivalent proportions of accumulated TCR-V and TCR-IV cell representatives from each transfer at the tumor-site (data not shown). These data further suggest that at 40 days post initiation of TCR-V cell-mediated therapy, SV11 tumors have not intrinsically changed, as tumors remain susceptible to both immunorecessive Tag epitope (TCR-V) and immunodominant Tag epitope (TCR-IV) mediated therapy. These results also imply that under these conditions, targeting the immunorecessive Tag epitope can be as effective as targeting the dominant epitope.

A proportion of TCR-V cells that persist long-term in the brains of SV11 mice following combinatorial immunotherapy are functional and actively proliferating

The inability for the persisting population of TCR-V cells from the initial round of combinatorial therapy to exert prolonged anti-tumor control in contrast to the enhanced effect of a second round of TCR-V cell infusion implied that the cells from the first transfer had lost functionality. In order to investigate this possibility, a group of SV11 mice was analyzed at 125
days of age (40 days following combinatorial treatment) and the persisting population of TCR-V cells was assessed for function and proliferative capacity, as determined by the production of IFNγ and in vivo incorporation of BrdU, respectively. In accordance with previous findings (Figure 39), high TCR-V cell numbers were maintained long-term in the brain (42% of CD8+ cells) (Figure 45A). Additionally, a population of TCR-V cells was detected in the spleen at this late time point (8% of CD8+ T cells) (Figure 45A). With respect to functionality of these cells, the results demonstrate that 20% of CD8+ cells from the brain made IFNγ in response to Tag-V peptide stimulation ex vivo (Figure 45B). This represented one-half of total Tetramer-V+ cells from the tumor-site that harbored effector function. A fraction of this population was actively proliferating, as 30% of IFNγ producing cells had also incorporated BrdU during the 48 hours prior to analysis (Figure 45B). These data indicate that not only did combinatorial treatment promote persistence of TCR-V cells at the tumor site of SV11 mice, a proportion of these cells were still actively proliferating and capable of displaying effector function.

**TCR-V cells isolated from SV11 tumors at 40 days post combinatorial immunotherapy do not proliferate in vitro**

Although a fraction of TCR-V cells were proliferating in vivo and capable of producing IFNγ at 40 days post initiation of combinatorial therapy, these cells were incapable of maintaining control over tumor progression. One caveat from the previous experiment lies in the inability to distinguish TCR-V cells that have accumulated in the brain for extended periods of time from potential recent immigrants from the periphery. Thus it remains possible that a large proportion of the TCR-V cells demonstrating functionality were not representative of TCR-V cells that had persisted long-term at the tumor site. A plausible explanation for the eventual failure of TCR-V cells to control SV11 tumors despite their high-frequency accumulation in the brain is that over time, persistent tumor-infiltrating TCR-V cells are eventually rendered non-functional, or anergic. The proliferative potential of the remaining TCR-V cells and in vivo cytotoxic function of the persistent TCR-V population at the tumor site remained unknown since all mice eventually died from tumor.

One method of addressing T cell anergy involves removal of T cells from the source of persistent antigen, and supplementation of IL-2 in order to assess whether the addition of cytokine can rescue proliferative capacity. IL-2 has been shown to delay the contraction phase
FIGURE 45

A Proportion of TCR-V Cells That Persist Long-Term
In the Brains of SV11 Mice Following Combinatorial Therapy
Are Functional and Actively Proliferating

TCR-V cells were adoptively transferred into 85 day-old SV11 mice. Mice were administered both αCD40 agonistic mAb the day before and after transfer and immunization with B6/V-only Tag cells 7 days post transfer (combinatorial immunotherapy). Mice were analyzed at 125 days of age (40 days following combinatorial immunotherapy) to assess the frequency and function of TCR-V cells that persisted in the spleen and brain.

A. A representative example of flow cytometric data for one of the mice that received combinatorial therapy is shown.

B. The persisting population of TCR-V cells in 125 day-old SV11 mice was assessed for function and proliferative capacity, determined by the production of IFNγ and in vivo incorporation of BrdU, respectively. SV11 mice that had received combinatorial therapy (αCD40 + day 7 immunization) were injected i.p. every 12 hours with 4 doses of 1 mg/mL BrdU 2 days prior to sacrifice (from 122 days old to 124 days old) in order to label proliferating cells. TCR-V cells were isolated from the spleens and brains the following day (125 days of age) and assayed for IFNγ production in response to Tag-V specific peptide stimulation via intracellular cytokine stain and analyzed for BrdU incorporation by CD8+/Tetramer-V+ cells via flow cytometry.
FIGURE 45

A Proportion of TCR-V Cells That Persist Long-Term
In the Brains of SV11 Mice Following Combinatorial Therapy
Are Functional and Actively Proliferating

A.

**SPLEEN**  
CD8  
Tag-V tetramer  
8%

**BRAIN**  
CD8  
Tag-V tetramer  
42%

B.

**SPLEEN**  
CD8  
IFNγ  
5%

**BrdU**

**BRAIN**  
CD8  
IFNγ  
20%

**BrdU**
of T cells as well as contribute to the reversal of anergy and tolerance (Beverly et al., 1992; Blattman et al., 2003; Schwartz, 2003). Thus, TCR-V cells were harvested from the tumors and spleens of SV11 mice at 40 days following receipt of the combinatorial treatment regimen (125 days old), labeled with CFSE, and placed separately into culture with either IL-2 alone, Tag-V peptide alone, or Tag-V peptide + IL-2. The cells were analyzed on day 6 of in vitro culture to assess TCR-V cell expansion and proliferation (Figure 46). The isolated populations of TCR-V cells represented 7% and 40% of total CD8+ cells present in the spleen and brain, respectively (Figure 46A). On day 6 of culture, TCR-V cells from the spleen had undergone minimal division in the presence of IL-2 only, but did not expand above the isolated frequency (5% of total CD8+ cells). However, when spleen isolates were administered Tag-V peptide in culture, TCR-V cells underwent significant proliferation, regardless of the presence of IL-2, and reached frequencies of 44% (Tag-V peptide, no IL-2) and 27% (Tag-V peptide + IL-2) of total CD8+ cells (Figure 46B). In contrast, while TCR-V cells represented the major proportion of the CD8+ cells in the brain (70% and 63% for Tag-V peptide only and Tag-V peptide + IL-2, respectively) these TCR-V cells had not undergone any rounds of proliferation, indicated by the non-dilution of the CFSE label (Figure 46B). Rather, it appeared as though both the tetramer- as well as tetramer+ cells had been lost from the culture, most likely due to apoptotic death. These results indicate that TCR-V cells isolated from the spleen and brain of SV11 mice at 40 days post combinatorial therapy behave very differently in their response to re-stimulation and IL-2 supplementation, and suggest that brain-infiltrating TCR-V cells exist in a state of anergy.

**TCR-V cells isolated from SV11 tumors do not proliferate in vitro upon αCD3 triggering and addition of supportive feeder cells**

In the assay for in vitro proliferation rescue, cultures were assessed at day 6 only to detect expansion and division of isolated TCR-V cells (Figure 46B) and it was determined that brain isolated TCR-V cells did not respond to stimulation. However, it remained possible that these TCR-V cells had divided and died early during culture, making them undetectable at day 6. Additionally, since TCR-V cells isolated from the spleen were cultured amidst other splenocytes, the presence of these additional non-TCR-V cell types may have provided an optimal environment for presentation of Tag-V peptide to TCR-V cells. In contrast, TCR-V cells from the brain lacked a similar cellular support matrix in the culture wells – a factor that could
FIGURE 46

TCR-V Cells Isolated From SV11 Tumors at 40 Days Post Combinatorial Immunotherapy Do Not Proliferate \textit{in vitro}

A. TCR-V cells were harvested from the tumors and spleens of SV11 mice at 40 days following receipt of the combinatorial treatment regimen (\(\alpha\)-CD40 + TCR-V cell adoptive transfer + day 7 immunization with B6/V-only Tag cells) and assessed for frequency by tetramer staining and are represented as \% of total CD8+ cells.

B. TCR-V cells isolated from SV11 tumors or spleens (10 pooled mice) at 40 days post combinatorial immunotherapy were labeled with 5 \(\mu\)M CFSE, and placed separately into 96-well culture wells (1x10^5 cells/well) with either IL-2 (10 U/mL) alone, Tag-V peptide (5\(\mu\)M) alone, or Tag-V peptide (5\(\mu\)M) + IL-2 (10 U/mL). The cells were analyzed on day 6 of \textit{in vitro} culture to assess TCR-V cell expansion and proliferation (dilution of CFSE) by staining for CD8 and Tetramer-V. Histograms showing CFSE were generated by gating on CD8+/Tet-V+ cells. \% on dot plots = \% of total CD8+ cells.
FIGURE 46

A. TCR-V CELLS ISOLATED AT 40 DAYS POST COMBINATORIAL THERAPY

<table>
<thead>
<tr>
<th>SPLEEN</th>
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<tbody>
<tr>
<td>CD8</td>
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<td>7% Tetramer-V</td>
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<tr>
<th>BRAIN</th>
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<tbody>
<tr>
<td>CD8</td>
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<tr>
<td>40% Tetramer-V</td>
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</table>

B. TCR-V CELLS AFTER 6 DAYS IN CULTURE

- **SPLEEN**
  - IL-2 only: 5%
  - Tag-V peptide only: 44%
  - Tag-V peptide + IL-2: 27%

- **BRAIN**
  - Tag-V peptide only: 70%
  - Tag-V peptide + IL-2: 63%
potentially have contributed to the lack of expansion and survival. Finally, the capacity of TCR-V cells to respond to non-specific stimulation remained unknown. Thus, the *in vitro* proliferation assay was repeated, with the addition of irradiated B6 splenocytes to the culture wells as a source of feeder cells. Culture conditions included Tag-V peptide + IL-2, IL-2 only, and plate-bound αCD3 + IL-2 (non-specific stimulation of the TCR). Cultures were assessed at days 2, 4, and 6 to detect frequency and proliferation of TCR-V isolated from the spleens vs. the brains of SV11 mice (*Figure 47*).

The input frequency of TCR-V cells isolated from SV11 spleens represented 10% of total CD8+ cells on day 0. Upon stimulation with Tag-V peptide TCR-V cells expanded to 45% of total CD8+ cells in the culture on day 6 (*Figure 47A*), with 38%, 59% and 97% of Tetramer-V+ cells proliferating on days 2, 4, and 6 respectively (*Figure 47B*). In the absence of stimulation (IL-2) only, minimal proliferation occurred and TCR-V cells did not expand above the input frequency. Upon triggering with αCD3, TCR-V cells underwent robust proliferation, representing 41%, 60%, and 81% of proliferating Tetramer-V+ cells on days 2, 4, and 6 respectively (*Figure 47B*). However, as αCD3 triggered all CD8+ cells in the culture to divide, the percentage of TCR-V cells as a fraction of total CD8+ cells was minimal by day 6 (*Figure 47A*).

An entirely different result was observed with TCR-V cells isolated from SV11 brains. The input frequency on day 0 represented 42% of total CD8+ cells in the culture (*Figure 47A*). However, regardless of the culture conditions, TCR-V cells did not demonstrate any dilution of the CFSE label by day 4, with only minimal division occurring by day 6 (*Figure 47B*). Correlative with the lack of proliferation was the significant decline in TCR-V cell frequency in all culture wells by day 6, representing 10%, 15%, and 1% of total CD8+ cells in response to Tag-V peptide, IL-2 only, and αCD3, respectively (*Figure 47A*). These data confirm the observations from Figure 34, and indicate that TCR-V cells that accumulate in the tumor site at 40 days post combinatorial therapy cannot be induced to proliferate *in vitro* – either specifically (Tag-V) or non-specifically (αCD3) – and highly suggest that these tumor-specific cells have been rendered anergic.
TCR-V Cells Isolated From SV11 Tumors Do Not Proliferate *in vitro* Upon αCD3 Triggering and Addition of Supportive Feeder Cells

A. TCR-V cells were harvested from the tumors and spleens of SV11 mice at 40 days following receipt of the combinatorial treatment regimen (α-CD40 + TCR-V cell adoptive transfer + day 7 immunization with B6/V-only Tag cells) and assessed for frequency as % of total CD8+ cells (day 0). TCR-V cells isolated from SV11 tumors or spleens (20 mice) were pooled, labeled with 5 µM CFSE, and placed separately into 96-well culture wells (1x10^5 cells/well) with irradiated B6 splenocytes (1x10^5 cells) as a source of feeder cells, and 10 U/mL IL-2. Culture conditions included 5 µM Tag-V peptide + IL-2 (specific TCR stimulation), IL-2 only, and plate-bound αCD3 (10 ug/mL) + IL-2 (non-specific TCR stimulation). Culture wells were assessed at days 2, 4, and 6 to detect frequency and proliferation of TCR-V cells.

B. Histograms are gated on CD8+Tet-V+ cells and bars indicate percentages of TCR-V cells that had undergone at least 2 rounds of proliferation, (indicated by dilution of the CFSE label) under the three *in vitro* culture conditions on days 2, 4, and 6.
FIGURE 47

A.

[Graphs showing the percentage of CD3+ cells in the spleen and brain over time for different treatments.]
FIGURE 47

B.

<table>
<thead>
<tr>
<th></th>
<th>Tag-V peptide + IL-2</th>
<th>IL-2 only</th>
<th>α-CD3</th>
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<tbody>
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<td><img src="image17" alt="Graph" /></td>
<td><img src="image18" alt="Graph" /></td>
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CFSE
TCR-V cells isolated from SV11 tumors early after combinatorial immunotherapy do proliferate *in vitro*

Given the striking difference between the proliferative response demonstrated by TCR-V cells isolated from the spleen versus cells isolated from the brains of SV11 mice at late time points following combinatorial immunotherapy, it remained possible that some factor related to the brain isolation protocol rendered any T cell recovered from the tumor site incapable of surviving *ex vivo* in culture. In order to address this question, TCR-V cells were isolated from SV11 spleens and brains at an earlier time point - 20 days following initiation of combinatorial immunotherapy (2 weeks following day 7 immunization). Recovered cells were labeled with CFSE and cultured *in vitro* for 6 days with irradiated B6 splenocytes as feeder cells, IL-2, and either Tag-V peptide or no peptide. Cultures were assessed at day 2, 4, and 6 to detect frequency and proliferation of TCR-V cells (*Figure 48*). On day 2, minimal proliferation had occurred and TCR-V cell frequencies resembled the original frequencies initially placed in the culture. TCR-V cells isolated from the spleen represented 12% and 9% of total CD8+ cells cultured with and without peptide, respectively. TCR-V cells isolated from the brain comprised 70% and 52% of total CD8+ cells cultured in peptide + IL-2 and IL-2 only, respectively (*Figure 48A*).

On the fourth day of culture, 44% of TCR-V cells isolated from SV11 spleens and cultured with Tag-V peptide/IL-2 had already begun to divide, representing 8% of total CD8+ cells (*Figure 48B*). This fraction appears incongruous with the amount of proliferation that had occurred, but may be explained by the proliferation of CD8+/tetramer- cells that also divided most likely due to the presence of cytokine. Thus, IL-2 alone did not induce significant proliferation of recovered TCR-V cells on this day of analysis. By day 4, 26% of brain-isolated TCR-V cells had begun to divide in response to Tag-V peptide, representing 55% of total CD8+ cells (*Figure 48B*). Minimal proliferation above non-specific division (22% of TCR-V cells) occurred in the absence of peptide, and relative frequency of TCR-V cells amidst non-specific expansion of tetramer- CD8+ cells dropped to 30% (*Figure 48B*).

By day 6 of *in vitro* culture, most of the spleen-isolated TCR-V cells stimulated with Tag-V peptide had completely lost the CFSE label indicating more than 7 rounds of proliferation, and represented 86% of total CD8+ cells. Furthermore, 70% of tumor-isolated TCR-V cells were actively dividing in response to peptide, representing 75% of total CD8+ cells (*Figure 48C*).
TCR-V cells were isolated from SV11 spleens and brains (12 mice) at 20 days following initiation of combinatorial immunotherapy (2 weeks following day 7 immunization). Recovered cells (1x10^5 cells/well) were pooled, labeled with 5µM CFSE and cultured in vitro for 6 days with irradiated feeder cells (1x10^5 cells/well), IL-2 (10 U/mL), and either 5µM Tag-V peptide or no peptide (IL-2 only).

Culture wells were assessed at (A.) day 2, (B.) day 4, and (C.) day 6 to detect frequency and proliferation of TCR-V cells. Histograms are gated on CD8+Tetramer-V+ cells and bars indicate percentages of TCR-V cells that had undergone at least 2 rounds of proliferation, (indicated by dilution of the CFSE label).

% on dot plots = % of Tetramer-V+ cells/total CD8+ cells
FIGURE 48

A. Tag-V peptide + IL-2

SPLEEN

12%

13%

9%

11%

DAY 2

BRAIN

70%

18%

52%

14%

Tetramer-V

CFSE

Tetramer-V

CFSE

B. Tag-V peptide + IL-2

SPLEEN

8%

44%

6%

28%

DAY 4

BRAIN

55%

26%

30%

22%

Tetramer-V

CFSE

Tetramer-V

CFSE

C. Tag-V peptide + IL-2

SPLEEN

40%

86%

30%

40%

DAY 6

BRAIN

75%

70%

60%

24%

Tetramer-V

CFSE

Tetramer-V

CFSE

IL-2 only
While minimal proliferation occurred in the absence of peptide, TCR-V cells persisted (60% of total CD8+ cells), amidst the loss of the CD8+ cells that initially expanded nonspecifically in response to cytokine (Figure 48C). Interestingly, although peptide-stimulated TCR-V cells isolated from tumors were proliferating and represented a significant proportion of total CD8+ cells by day 6, these cells did not accumulate in the CFSE\textsuperscript{low} channel, suggesting that these cells are either dividing more slowly or die after several rounds of proliferation.

These results confirm that TCR-V cells isolated from SV11 tumors 20 days following adoptive transfer and initiation of combinatorial therapy are capable of proliferating in response to Tag-V peptide \textit{ex vivo}, leading to the greatest expansion between days 4 and 6 in culture. Additionally, even in the absence of peptide, brain-isolated TCR-V cells persisted in culture, indicating that these cells have been imprinted with a signal to survive. However, this trend is not observed at later time points post therapy (Figure 47). Together with the results presented in Figure 47, these data suggest that in the window between 20 days and 40 days post combinatorial therapy, tumor-infiltrating TCR-V cells are rendered anergic, incapable mounting an \textit{in vitro} proliferative response to antigen.

**TCR-V cells isolated from the brains of SV11 mice following combinatorial immunotherapy do not exhibit \textit{in vivo} cytotoxic function**

The inability for TCR-V cells isolated from SV11 tumors to proliferate \textit{in vitro} (Figure 46 and Figure 47) and the apparent loss of anti-tumor control at late time points following combinatorial therapy (Figure 44) strongly suggested that tumor-infiltrating TCR-V cells are eventually rendered anergic. It has been demonstrated in other systems that following removal of tolerant T-CD8 memory cells from the source of persistent antigen (virus-vaccinated mice + peptide boost) and transfer into an \textit{in vivo} antigen-free environment, T-CD8 functional capacity was regained as demonstrated by the ability of T-CD8 to reject an otherwise lethal tumor challenge (den Boer et al., 2004). These reports indicated that while memory T-CD8 responses may be functionally incapacitated, they are not irreversibly tolerized by persistent systemic antigen, as memory T cells quickly regain effector function upon removal of the antigen.

To this end, the functionality of TCR-V cells harvested from the tumors and spleens of SV11 mice at 40 days following receipt of the combinatorial treatment regimen was assessed.
TCR-V cells were isolated from SV11 tumors and spleens at 40 days post initiation of treatment, and equal numbers (2x10⁶ cells) of either spleen-isolated or brain-isolated TCR-V cells were transferred into B6 hosts. Recipient mice were immunized the following day in order to reactivate TCR-V cells and were assayed seven days later for their ability to kill Tag-V pulsed targets in vivo (Figure 49). Control mice included B6 mice that were either given a primary immunization or prime/boost immunization to activate endogenous Tag-V specific T-CD8. In addition, B6 mice that had been adoptively transferred with naïve TCR-V cells and immunized at the same time points as the SV11 mice were analyzed (Figure 49).

B6 mice that had received only a primary immunization 7 days earlier demonstrated meager expansion (1.8% of total CD8+ CD45.1- cells) and no detectable cytotoxicity toward target cells (Figure 50 – first panel). This result was not surprising, since the endogenous Tag-V specific response does not peak until 9 days post-immunization. In B6 mice that had been primed and boosted, Tag-V cells expanded to 5.6% of total CD8+CD45.1- cells and demonstrated 70% specific killing of Tag-V pulsed target cells (Figure 50 – second panel). In B6 mice that had received naïve TCR-V cells + immunization, TCR-V cells expanded to 7% of CD8+CD45.1+ cells and exhibited 84% specific cytotoxicity (Figure 50 – third panel). These results indicated that immunization could activate Tag-V specific T-CD8 populations (both CD45.21 endogenous and CD45.1+ TCR-Tg) in B6 mice that harbored in vivo cytotoxic function.

In B6 mice that had received spleen-isolated TCR-V cells from SV11 mice at 40 days post combinatorial therapy, TCR-V cells expanded to 3% of total CD8+ cells and demonstrated 40% specific killing of Tag-V pulsed targets (Figure 50 – bottom panels). In contrast, tumor-infiltrating TCR-V cells did not expand in response to immunization following isolation from SV11 brains and transfer into B6 hosts. TCR-V cells represented a meager 1% of total CD8+ cells and harbored no cytotoxic function toward target cells (Figure 50 – fourth panel). Thus while TCR-V cells isolated from SV11 spleens at late time points post combinatorial therapy appeared to be functionally compromised (40% killing) compared with TCR-V cells isolated from B6 primary hosts (84% killing) or the endogenous population of Tag-V cells primed and boosted in B6 mice (70%), TCR-V cells isolated from SV11 tumors were functionally incompetent (0% killing). Together with the results from the previous section, these data further support the prediction that tumor-infiltrating TCR-V cells become compromised in their ability to expand,
FIGURE 49

Protocol For Cytotoxic Assessment of TCR-V Cells Isolated From SV11 Tumors 40 days Post Combinatorial Immunotherapy

A. The functionality of TCR-V cells harvested from the tumors and spleens of SV11 mice at 40 days following receipt of the combinatorial treatment regimen was assessed. TCR-V cells were isolated from SV11 tumors and spleens at 40 days post initiation of treatment.

B. TCR-V cells were isolated from SV11 tumors and spleens (pooled from 20 mice) at 40 days post initiation of combinatorial treatment. The frequency of Tetramer-V/CD8+ cells was assessed flow cytometrically, and the appropriate number of total spleen-isolated or brain-isolated TCR-V cells were transferred into B6 hosts such that mice received $2 \times 10^6$ transgenic cells. Recipient mice were immunized i.p. the following day with $5 \times 10^7$ B6/V-only Tag cells in order to reactivate TCR-V cells and were assayed seven days later for their ability to kill Tag-V pulsed targets in vivo. Target cells included $2.5 \times 10^6$ Tag-V (5µM) peptide-pulsed splenocytes labeled with 5µM CFSE and $2.5 \times 10^6$ Flu-NP (5µM) peptide-pulsed splenocytes labeled with 0.5µM CFSE. Target cells were combined and injected into B6 i.v. 7 days post re-transfer + immunization. Control mice included B6 mice that were either given a primary immunization or prime/boost immunization to activate endogenous Tag-V specific T-CD8 as well as B6 mice that had received naïve TCR-V cell adoptive transfer + immunization. All B6 control mice were assessed for specific cytotoxicity of target cells 7 days following the final immunization.
FIGURE 49

A. TCR-V cell isolation from SV11 mice

Day 0
TCR-V cell adoptive transfer + a-CD40 mAb

Day 7
Tag-V immunization
wait 33 days

Day 40
Isolate TCR-V cells from SPLEEN and BRAIN for adoptive transfer into B6 hosts

B. In Vivo Cytotoxicity Assay

After 7 days
Prepare targets pulsed with:
Control peptide OR Tag-V peptide

Inj ect labeled targets

CFSE

Analysis
specific loss of labeled targets

SV11 donors

SPLEEN- isolated TCR-V cells
or
BRAIN-isolated TCR-V cells
or
naive TCR-V cells

B6 hosts

Tag-V immunization

wait overnight
FIGURE 50

TCR-V Cells Isolated From SV11 Tumors At 40 Days Following Combinatorial Immunotherapy Do Not Exhibit in vivo Cytotoxicity

The expansion and in vivo cytotoxic function of TCR-V cells isolated from the tumors and spleens of SV11 mice (pooled from 20 mice) that had received combinatorial immunotherapy was assessed following isolation and re-transfer into B6 hosts followed by immunization with B6/V-only Tag cells. Equal numbers (2x10^6 cells) of either spleen-isolated or brain-isolated TCR-V cells were transferred into B6 hosts. Recipient mice were immunized the following day with B6/V-only Tag tumor cells in order to reactivate TCR-V cells and were assayed seven days later for their ability to kill Tag-V pulsed targets in vivo. Equal numbers of Tag-V peptide or Flu-NP peptide pulsed target cells (2.5x10^6 each) were labeled with 5 µM or 0.5 µM concentrations, respectively, of CFSE, and intravenously injected into B6 hosts. Mice were assessed the next day for frequency of TCR-V cells in the spleen and for specific loss of Tag-V peptide pulsed targets.

Experimental and control recipients included (3 mice/group): (i.) B6 mice given primary immunization 7 days before target injection; (ii.) B6 mice given prime and boost immunization 14 and 7 days, respectively before target injection; (iii.) B6 mice given naïve TCR-V cells+immunization 7 days before target injection; (iv.) B6 mice given TCR-V cells isolated from SV11 tumors + immunization 7 days before target injection; and (v.) B6 mice given TCR-V cells isolated from SV11 spleens + immunization 7 days before target injection. The control percentages of Tag-V peptide-pulsed and Flu-NP-pulsed target cells are shown (top panel), as well as the frequency of TCR-V cells expanded in recipient mice from each condition (left panels), the source of Tag-V specific cells (CD45.1+ = TCR-V cells; CD45.1- = endogenous Tag-V specific cells) (middle panels); and final specific cytotoxicity of Tag-V specific T-CD8 toward Tag-V pulsed target cells (right panels).

The following formula was used to determine the percentage of specific killing:

\[%\ lysis = \frac{1 - \text{(ratio unprimed/ratio primed)}}{\text{100}}\], \text{where ratio} = \left(\frac{\% \text{ of CFSE}^{\text{low}} \text{ cells}}{\% \text{ of CFSE}^{\text{high}} \text{ cells}}\right).\]
FIGURE 50

TCR-V Cells Isolated From SV11 Tumors at 40 Days Following Combinatorial Immunotherapy Do Not Exhibit in vivo Cytotoxicity

control
targets

CFSE

% cytotoxicity

B6
primary immunization

B6
primary+ booster immunization

B6
naive TCR-V cells + booster immunization

B6
SV11 BRAIN-isolated TCR-V cells+ booster immunization

SV11
SV11 SPLEEN-isolated TCR-V cells+ booster immunization

CD8
Tetramer-V

Flu-NP Tag-V peptide peptide

Flu-NP Tag-V peptide peptide

Flu-NP Tag-V peptide peptide
both *in vitro* and *in vivo*, which may partially explain their limited cytotoxic function and eventual failure to maintain control over tumor progression.

**TCR-V cells that persist long-term in the periphery of SV11 mice following combinatorial immunotherapy exhibit *in vivo* cytotoxic function**

Although TCR-V cells isolated from SV11 tumors at 40 days post combinatorial therapy were limited in their ability to divide *in vitro* (Figures 46 and 47) and exhibited *in vivo* functionality (Figure 50), it was also observed that at this same time point that a fraction of brain-infiltrating TCR-V cells incorporated BrdU *in vivo* and produced Tag-V specific IFNγ *ex vivo* (Figure 45). Additionally, TCR-V cells isolated from SV11 spleens differed dramatically from cells isolated from SV11 tumors in respect to their *in vitro* proliferative potential (Figures 46 and 47). Thus, it was possible that the portion of brain-resident cells shown to be dividing and functional (Figure 45) represented TCR-V cells that had recently immigrated from the periphery.

The only way to assess cytotoxic function of brain-resident cells was to remove them from SV11 mice and transfer them into new B6 hosts (Figures 49 and 50), as there is no feasible method for introducing target cells directly into the brain tumor. However, the population of TCR-V cells persisting in the periphery of SV11 mice at late time points can be assessed directly in this manner. Thus, to address the possibility that TCR-V cells in the brain differ from those in the periphery, it was important to determine whether peripheral TCR-V cells demonstrated cytotoxic function in SV11 hosts, eliminating the need for isolation and re-transfer.

Therefore, at 125 days of age (40 days following combinatorial treatment) some SV11 mice and B6 mice received a booster immunization with B6/V-only Tag cells, while the remaining SV11 mice did not receive a booster. One week later, mice were intravenously injected with differentially labeled CFSE-labeled target cells pulsed with Tag-V peptide or Flu-NP control peptide. The following day, spleens were harvested and assessed for frequency of TCR-V cells and frequency of remaining target cells in order to determine specific killing of Tag-V peptide pulsed cells by the peripheral population of persisting TCR-V cells (Figure 51).

In SV11 mice that did not receive a booster immunization, TCR-V cells represented 6% of total CD8+ cells in the spleen, which correlated with 91% specific cytotoxicity toward Tag-V pulsed target cells (Figure 51 – first panel). In both SV11 and B6 mice that received a booster,
FIGURE 51

TCR-V Cells That Persist Long-Term in the Periphery of SV11 Mice
Following Combinatorial Immunotherapy
Demonstrate in vivo Cytotoxicity

TCR-V cells were adoptively transferred into 85 day-old SV11 mice and B6 transgene
negative littermates. Mice were administered both αCD40 agonistic mAb the day before
and after transfer and immunization with B6/V-only Tag cells 7 days post transfer. At
125 days of age (40 days following combinatorial treatment) some mice received a
booster immunization with B6/V-only Tag cells. One week later mice were intravenously
injected with equal numbers of Tag-V peptide (5 μM) or Flu-NP (5 μM) peptide-pulsed
target cells (2.5x10^6 each), labeled with 5 μM or 0.5 μM concentrations, respectively, of
CFSE. The following day, spleens were harvested and assessed for frequency TCR-V
cells and of remaining target cells in order to determine specific killing of Tag-V peptide
pulsed cells by the peripheral population of persisting TCR-V cells.
Each experimental group consisted of 3 mice. Dot plots (left panels) indicate the
frequency of Tetramer-V+CD8+ cells/total CD8+ splenocytes in a representative mouse
from each experimental set. The control percentages of Tag-V peptide-pulsed and Flu-
NP-pulsed target cells (top panel) and specific cytotoxicity towards Tag-V pulsed target
cells (right panels) are shown.

The following formula was used to determine the percentage of specific killing: % lysis =
[1- (ratio unprimed/ratio primed) x 100], where ratio = (% of CFSE^{low} cells/% of CFSE^{high}
cells).
FIGURE 51

TCR-V cells in the periphery of SV11 mice are cytotoxic in vivo at 40 days following combinatorial immunotherapy.
TCR-V cells expanded to 25% and 22% of CD8+ cells, respectively. These frequencies corresponded to 100% and 98% specific cytotoxicity (Figure 51 – middle and bottom panels). Afore-mentioned anatomical constraints obviated the ability to address the in vivo cytotoxic function of TCR-V cells at the tumor site. However, these results indicate that TCR-V cells that persist in the periphery of SV11 mice 40 days post combinatorial therapy as well as memory TCR-V cells in the periphery of B6 mice respond to immunization. Importantly, these cells retain significant cytotoxic function in vivo. These data indicate that peripheral TCR-V cells differ from tumor-infiltrating TCR-V cells, further suggesting that the latter are subject to tolerance induction by persistent Tag expression. The peripheral TCR-V cell population is thus implicated as a pool of T-CD8 cells from which functional tumor-specific cells may be recruited for trafficking into the brain throughout the course of disease progression.

The phenotype of TCR-V cells in SV11 tumors following combinatorial therapy differs significantly from peripheral TCR-V cells

The difference in both proliferative potential (Figure 46 and Figure 47) and cytotoxic function (Figures 50 and 51) of TCR-V cells that accumulated in SV11 brains compared to TCR-V cells recovered from SV11 spleens following combinatorial therapy suggested that tumor-infiltrating TCR-V cells had been altered within the tumor microenvironment. Maintenance of the peripheral T cell compartment is significantly influenced by cytokines, in particular IL-7 and IL-15 (Tuma and Pamer, 2002). Therefore, the expression of several cell surface markers reflective of T cell activation and memory status were assessed on TCR-V cells present within the tumor stroma following combinatorial therapy and compared with the phenotype of TCR-V cells that remained in the periphery (Figure 52).

L-selectin

The lymph node homing receptor L-selectin (also known as CD62L) is expressed on both naïve and central memory cells, and is down-regulated upon activation and commitment to the effector memory cell subset. At early time points following adoptive transfer and immunization of SV11 mice, L-selectin is down-regulated on a large proportion of TCR-V cells from the spleen (Figures 17 and 23), indicative of recent activation. TCR-V cells isolated from the brain are always L-selectinlo, suggesting that only activated cells are permitted access to the brain (Figure 23 and observed data not shown). When the L-selectin expression status of
The expression of cell surface markers reflective of T cell activation and memory status were assessed on TCR-V cells present within the tumor stroma following combinatorial therapy and compared with the phenotype of TCR-V cells that remained in the periphery. Tumors and spleens of SV11 mice and spleens of B6 mice that had received TCR-V cell adoptive transfer + CD40 conditioning + day 7 immunization were isolated at 40 days post initiation of therapy (125 days of age) and frequency of TCR-V cells was assessed by staining for CD8 and Tetramer-V. Dot plot % = % of CD8+/Tetramer-V+ cells/total CD8+ cells. Cells were also stained for expression of L-selectin, CD122, IL-7R, and Ly6C. Histograms represent fractions of gated CD8+/Tetramer-V+ cells that stained positive for the markers.
FIGURE 52

The Phenotype of TCR-V Cells in SV11 Tumors Following Combinatorial Therapy Differs Significantly From Peripheral TCR-V Cells
TCR-V cells at this late time point was assessed, it was found that tumor-infiltrating cells maintained their L-selectin<sup>lo</sup> phenotype (Figure 52 - bottom panel). However, in the spleens of both B6 (middle panel) and SV11 mice (top panel), a large proportion of TCR-V cells had regained expression of L-selectin. For B6 mice (17 % L-selectin<sup>lo</sup> TCR-V cells), this would be expected, as the cells had not experienced antigen exposure for 40 days and these cells likely represented the central memory population of TCR-V cells (Wherry et al., 2003b). In SV11 mice, a larger proportion of TCR-V cells were L-selectin<sup>lo</sup> (29 % L-selectin<sup>lo</sup> TCR-V cells), which might be expected from the continuous presence of Tag in the tumor-draining CLN. Importantly, the L-selectin phenotype of tumor-infiltrating TCR-V cells differed significantly from that of TCR-V cells remaining in the periphery, in that TCR-V cells in the periphery had re-acquired the expression of a receptor normally characterized on memory T-CD8 cell subsets.

**CD122 (IL-2βR) and CD127 (IL-7R)**

The IL-2R is composed of three chains, α (CD25), β (CD122), and γ (CD132), of which only the α chain is specific for IL-2. Signaling takes place mainly via CD122 (Minami, 1993). Activated T cells express the high affinity receptor composed of all three chains. Additionally, CD122 is one of the critical subunits of IL-15R and a crucial component of both IL-2 and IL-15-mediated signaling. IL-15 shares many activities exerted by IL-2, including the stimulation and expansion of T cells owing to the fact that the receptors for IL-15/IL-2 share the IL-2Rβ chain (CD122), and memory T cells bear high levels of this receptor (Nelson, 1998 Cho, 1999). However, in murine models, IL-15 unlike IL-2 fails to significantly activate the apoptotic pathways when stimulating T cells, suggesting that IL-15 promotes the establishment of long-term memory T cells. Thus, IL-15 has been implicated as a crucial growth factor in maintenance specifically of memory T cells (Li et al., 2001b; Ma et al., 2000), whereas IL-2 inhibits memory survival (Bennett et al., 2003; Liu et al., 2002a; Weng et al., 2002).

Interleukin 7 (IL-7) is a non-redundant cytokine important for T-lymphocyte development and function (Hofmeister et al., 1999). The IL-7R (CD127) is down-regulated on activated T cells, suggesting the essential role of IL-7 in maintenance of resting naïve and memory T cells (Hofmeister et al., 1999; Kaech et al., 2003; Schluns et al., 2000; Tan et al., 2001).

The expression of CD122 and CD127 as related to the maintenance of TCR-V cells in SV11 mice following combinatorial immunotherapy was therefore analyzed (Figure 52). TCR-V
cells isolated from SV11 spleens and tumors were compared to TCR-V cells isolated from B6 spleens at 40 days post initiation of therapy. In SV11 spleens, a distinct portion of 78% of TCR-V cells demonstrated up-regulation of CD122 (Figure 52 – top panel). In B6 spleens, CD122 expression was less intense, most likely related to the absence of persistent antigen (Figure 52 – middle panel). In sharp contrast to CD122 expression by TCR-V cells in SV11 spleens, TCR-V cells in SV11 brains demonstrated down-regulation of the receptor, with only 16% of TCR-V cells staining positive for CD122, suggesting that these cells were perpetually activated by antigen within the tumor (Figure 52 – bottom panel).

A similar trend was observed regarding the expression of CD127. In both SV11 and B6 spleens, CD127 was up-regulated, with 72% and 77% of TCR-V cells expressing the receptor, respectively (Figure 52 – top and middle panels). However, in SV11 brains, a small fraction of 20% of TCR-V cells demonstrated CD127 expression, further indicating that these tumor-resident TCR-V cells were persistently activated (Figure 52 – bottom panel). These data suggest that memory TCR-V cell status is established in the periphery, possibly providing a feeder source for TCR-V cells that traffic to and infiltrate SV11 tumors.

**Ly6C**

Ly6C is a hematopoietic cell differentiation Ag found on a subset of CD8+ T cells in the periphery. It has shown to be involved in target cell killing by T-CD8 cells, augmentation of TCR-mediated IL-2 and IFNγ production in T-CD8 cells, and regulates T-CD8 cell homing in vivo (Havran et al., 1988; LeClair et al., 1987; Walunas et al., 1995). Cross-linking of Ly6C causes clustering of LFA-1 (CD11α/CD18) on the surface of T-CD8, which significantly augments lymphocyte adhesion to endothelium, and is inhibited by antagonistic mAbs that block LFA-1 function. Furthermore, upon in vitro cross-linking and during in vivo homing into lymph nodes, Ly6C is transiently lost from the cell surface but becomes re-expressed on lymph node-resident T-CD8 cells. Ly6C induction of LFA-1 clustering and re-expression after signaling-associated down-regulation has been implicated in regulation of T-CD8 cell homing into lymph nodes and in subsequent steps of T-CD8 cell activation and effector function (Jaakkola et al., 2003). Given its multiple associations with T-CD8 cell functions, it was hypothesized that if tumor-infiltrating TCR-V cells are truly rendered non-responsive, they might differ in expression of the Ly6C marker compared to the phenotype of TCR-V cells in the spleen.
While Ly6C expression on TCR-V cells in both SV11 and B6 spleens was heterogeneous, all TCR-V cells demonstrated positive staining for the marker, suggesting that these cells were capable of responding to antigen stimulation (**Figure 52 top and middle panels**). In SV11 tumors, 2 distinct populations were observed, with 60% of TCR-V cells expressing intermediate to high levels of Ly6C, and 40% demonstrating absolutely no expression of the marker (**Figure 52 – bottom panel**). This latter population might represent recently activated TCR-V cells that had subsequently down-regulated Ly6C during signaling. Combined with the observation that only a fraction of brain-isolated TCR-V cells proliferated *in vivo* and were capable of IFNγ production (Figure 45), these data suggest that despite the large accumulation of TCR-V cells in SV11 tumors, only a subset of these cells are activated at a given moment in time.

**TCR-V cells isolated from the brains of SV11 mice following combinatorial immunotherapy irreversibly up-regulate the inhibitory receptor PD-1**

In response to antigen, T-CD8 cells up-regulate the inhibitory receptor, PD-1 in order to protect against uncontrolled T cell activation. In the context of chronic antigen expression, antigen-specific T-CD8 cells have been reported to exhibit persistent up-regulation of PD-1, leading to tolerance. In order to assess the potential role of PD-1 in ultimate anergic rendering of tumor-infiltrating TCR-V cells, the phenotype of TCR-V cells isolated from spleens and brains of SV11 mice was analyzed at 40 days post combinatorial therapy (**Figure 53**). It was found that a portion of spleen-resident TCR-V cells (30%) expressed PD-1, perhaps due to persistent antigen present in the tumor-draining CLN (**Figure 53A**). Upon analysis of tumor-infiltrating TCR-V cells, virtually the entire population of TCR-V cells that had accumulated at the tumor site post combinatorial therapy expressed high levels of the inhibitory receptor PD-1 in comparison to the lack of PD-1 expression on Tetramer-/CD8+ cells in the brain (blue histogram overlay) (**Figure 53A**).

In order to address the ability of TCR-V cells to down-regulate PD-1, TCR-V cells isolated from the spleens and brains of SV11 mice at 40 days post combinatorial therapy were cultured for 6 days with (i.) Tag-V peptide + IL-2, (ii.) IL-2 only (absence of antigen), and (iii.) with plate-bound αCD3 + IL-2. Cultures were assessed on days 2, 4, and 6 to address down-regulation of PD-1 (**Figure 53B**). TCR-V cells from SV11 spleens and cultured with Tag-V
FIGURE 53

TCR-V Cells Isolated from SV11 Tumors Following Combinatorial Immunotherapy Irreversibly Up-regulate the Inhibitory Receptor PD-1

A. TCR-V cells were harvested from the tumors and spleens of 14 SV11 mice at 40 days following receipt of the combinatorial treatment regimen (α-CD40 + TCR-V cell adoptive transfer + day 7 immunization with B6/V-only Tag cells) and assessed for frequency of TCR-V cells by staining for Tetramer-V and CD8 and for expression of PD-1. Histograms represent gated populations of Tetramer-V+/CD8+ cells and indicate the % of TCR-V cells expressing PD-1. Dot plots and histograms are examples from the tumor and spleen of a representative SV11 mouse at this time point.

B. The TCR-V cells isolated from the spleens and brains of 14 SV11 mice at 40 days post combinatorial therapy (Part A) were stained separately for Tetramer-V and CD8 to ensure consistent TCR-V cell frequencies, and cells from 12 of these mice were pooled. TCR-V cells isolated from SV11 tumors or spleens were placed separately into 96-well culture wells (1x10^5 cells/well) with irradiated B6 splenocytes (1x10^5 cells) as a source of feeder cells, and 10 U/mL IL-2. Culture conditions included 5 μM Tag-V peptide + IL-2 (specific TCR stimulation), IL-2 only, and plate-bound αCD3 + IL-2 (non-specific TCR stimulation). Culture wells were assessed on days 2, 4, and 6 to detect down-regulation of PD-1 by TCR-V cells. Gated populations of Tetramer-V+/CD8+ cells were assessed for the mean fluorescence intensity (MFI) of PD-1 expression as represented by the bar graphs (1 bar = 1 sample well).

C. Representative flow cytometric histogram of PD-1 expression by spleen-isolated and tumor-isolated TCR-V cells (gated on Tet-V+CD8+ cells) on day 6 of ex vivo culture in IL-2 only. % = % of PD-1+ cells/total gated TCR-V cells.
FIGURE 53

A.

CD8

6%

30%

Spleen

BRAIN

red: Tetramer-V+CD8+ cells
blue: Tetramer-V-/CD8+ cells

48%

98%

PD-1

B.

Day 2

TCR-V cells isolated from SV11 mice at 40 days post combinatorial therapy after 6 days in antigen free culture + IL-2

Day 4

Day 6

C.

Spleen

13%

BRAIN

95%

PD-1
peptide retained high level expression of PD-1 through day 4, but expression levels had dropped by one-half mean fluorescence intensity by day 6. In response to αCD3, spleen-isolated cells never down-regulated PD-1, due to persistent TCR stimulation (Figure 53B). In the absence of stimulation (IL-2 only), spleen-isolated TCR-V cells down-regulated PD-1 by day 4, with a further decrease observed by day 6 (Figure 53B), representing 13% of TCR-V cells exhibiting high-level PD-1 expression (Figure 53C). This represented a decline from 30% of TCR cells expressing PD-1 on the day of isolation (Figure 53A). In contrast, TCR-V cells isolated from SV11 tumors did not down-regulate PD-1 under any of the culture conditions (Figure 53B), with 95% of TCR-V cells demonstrating persistent PD-1 expression by day 6 of culture (Figure 53C). These data implicate up-regulation of PD-1 as a candidate for the anergic rendering of tumor-infiltrating TCR-V cells in SV11 mice, and suggest that TCR-V cells at the tumor site are irreversibly tolerized.

**Following combinatorial immunotherapy, SV11 choroid plexus tumors do not express B7-H1 (PD-1 ligand)**

The investigations of potential TCR-V cell anergy found that following combinatorial therapy, TCR-V cells that accumulated in SV11 tumors irreversibly expressed high levels of the inhibitory receptor, PD-1 (Figure 53). The two ligands for PD-1 are PD-L1 (B7-H1) and PD-L2 (B7-DC), which bind to PD-1, but not to other members of the CD28 family and exhibit differential patterns of expression, especially during diseased states (Dong et al., 1999; Freeman et al., 2000; Latchman et al., 2001). PD-L1 (B7-H1) is a cell surface glycoprotein belonging to B7 family of co-stimulatory molecules. Ligation of the PD-1 receptor by B7-H1 inhibits proliferation and cytokine production by activated T cells (Freeman et al., 2000). B7-H1 is expressed on resting T cells, B cells, DCs, and macrophages, and is further up-regulated upon activation (Nakazawa et al., 2004; Schoop et al., 2004; Yamazaki et al., 2002). B7-H1 transcripts are found in many non-lymphoid organs, including heart, placenta, skeletal muscle, liver, pancreas, spleen, and lung. Importantly, expression of B7-H1 is up-regulated in DCs and tumors following exposure to IFNγ (Dong et al., 1999; Freeman et al., 2000; Latchman et al., 2001).

It is possible that the engagement of PD-1 by a brain-resident cell type expressing B7-H1 is responsible for the ultimate anergic phenotype of TCR-V cells following combinatorial
therapy. Thus, the potential expression of B7-H1 by choroid plexus tumors as a candidate ligand for PD-1 expressed on TCR-V cells was addressed. In order to assess the phenotype of SV11 choroid plexus tumors, it was necessary to isolate choroid plexus cells and determine a method to allow for the specific identification of tumor cells amidst the remaining brain parenchyma. SV11 choroid plexus tumors express significant levels of the high-affinity folate receptor compared to normal choroid plexus tissue that can be specifically detected with antibody (Patrick et al., 1997). Additionally, since choroid plexus tissue is not bone marrow-derived, tumor cells do not express CD45. Therefore, detection of CP cells in both SV11 mice and B6 mice was accomplished through collagenase digestion of either untreated SV11 tumors, progressive (large) tumors from SV11 mice that had received combinatorial therapy, or B6 whole brains, and co-staining with antibodies for folate receptor, CD45.2, and B7-H1 for flow cytometric analysis (Figure 54A).

SV11 tumors in mice that received no treatment as well as SV11 mice whose tumors progressed in spite of combinatorial therapy were enriched for choroid plexus tumor cells that expressed high levels of folate receptor and were negative for CD45.2. Tumor-free B6 mice expressed detectable, albeit low numbers of folate receptor+ cells in the brain, consistent with normal choroid plexus tissue (Figure 54A). However, folate receptor+CD45.2- SV11 tumor cells demonstrated no expression of B7-H1, indicating that if PD-1 does play an inhibitory role in TCR-V cell mediated immunotherapy, the partner ligand B7-H1 is not present on the tumor cells themselves (Figure 54A). The potential expression of the alternative PD-1 ligand – B7-DC – however, remains undetermined.

Following combinatorial immunotherapy, SV11 choroid plexus tumors do express high levels of MHC class I

Another possible explanation for the eventual failure of TCR-V cells to control SV11 tumors despite their accumulation in high numbers in the brain is that the tumor and/or tumor microenvironment has been altered such that TCR-V cells can no longer recognize the tumor or can no longer functionally control tumor growth within the tumor milieu. One mechanism used by tumors to escape recognition by tumor-specific T cells is the loss of MHC class I expression (Ferrone and Marincola, 1995; Vitale et al., 1998; Wang et al., 1996). To address this possibility, tumors from SV11 mice that had received either no treatment or combinatorial
FIGURE 54

Following Combinatorial Immunotherapy, SV11 Choroid Plexus Tumors Do Not Express B7-H1 (PD-1 ligand), But Do Express High Levels of MHC Class I

A. Choroid plexus tissue was isolated from untreated SV11 tumors, progressive (large) tumors from SV11 mice that had received combinatorial therapy, and B6 whole brains by collagenase digestion (4 mice/group). Cells were co-stained for cell surface expression of folate receptor, CD45.2, and B7-H1 in preparation for flow cytometric analysis. Error bars indicate standard deviation from the mean.

B. Isolated folate receptor+/CD45.2- choroid plexus cells (from A.) were also stained for cell surface expression of MHC class I molecules H-2D\textsuperscript{b} and H-2K\textsuperscript{b} and analyzed via flow cytometry. Data is presented as mean fluorescence intensity (MFI) of MHC class I molecule expression by gated folate receptor+/CD45.2- cells. Error bars indicate standard deviation from the mean.
FIGURE 54

SV11 Choroid plexus Tumors Do Not Express B7-H1 (PD1 ligand), But Do Express High Levels of MHC Class I

A. 

B. MHC class I expression by choroid plexus

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315
therapy as well as brains from B6 mice were digested with collagenase to isolate choroid plexus cells, and stained for folate-receptor, CD45.2 and MHC class I (H-2K\textsuperscript{b} and H-2D\textsuperscript{b}) (Figure 54B).

The results show that tumors from untreated SV11 mice expressed levels of both H-2K\textsuperscript{b} and H-2D\textsuperscript{b} similar to MHC class I levels expressed by normal B6 choroid plexus cells (Figure 54B). By comparison, SV11 mice that had received combinatorial therapy expressed approximately 75% higher levels of both MHC class I molecules, with H-2D\textsuperscript{b} (the restricting MHC for Tag-V epitope) demonstrating the highest MFI (Figure 54B). This result is understandable, given that tumor-infiltrating TCR-V cells (Figure 45) demonstrate the capacity to produce IFN\textgamma - a molecule known to induce up-regulation of MHC expression. Thus, these data indicate that loss of MHC class I is not a mechanism employed by SV11 tumors as a means to escape recognition by TCR-V cells.

Multiple bone marrow-derived APC populations infiltrate SV11 tumors following combinatorial immunotherapy

It has been shown in other systems that during tumor progression, various bone marrow-derived APC cell types accumulate within the tumor stroma, including CD11c+ , CD11b+ , and Gr-1+ cells (Bronte et al., 1998; Gabrilovich et al., 2001; Kusmartsev et al., 2005; Kusmartsev et al., 2000). The presence of infiltrating APC populations may alter antigen presentation at the tumor site as well as affect T cell responses as a result of cytokine production (Zou, 2005). In order to investigate the potential role of tumor-infiltrating APC to dampen the TCR-V cell response to SV11 tumors following combinatorial therapy, the presence of various APC populations at the tumor site was analyzed by staining for known markers of different APC cell types and compared to the APC population in B6 brains (Figure 55).

A distinct population of CD11c+CD45+ cells, consisting of 20% of total tumor stromal cells, was detected in SV11 tumors (Figure 55). This CD45+CD11c+ bone marrow-derived population most likely representing DCs, could be sub-divided into 2 subsets according to the expression of CD11b. One-third exhibited expression of CD11b, while the remaining two-thirds of CD45+CD11c+ cells did not express CD11b. In contrast, very few bone marrow-derived CD11c+ cells or CD11c+CD11b+ were found in B6 brains (Figure 55). In order to address the frequency of macrophages within the tumor stroma, tumors and brains were stained with CD11b
FIGURE 55

Multiple Bone Marrow-Derived APC Populations Infiltrate SV11 Tumors Following Combinatorial Immunotherapy

Choroid plexus tissue was isolated from progressive (large) tumors from SV11 mice that had received combinatorial therapy, and B6 whole brains via collagenase digestion (4 mice/group). Resulting cell suspensions were co-stained for cell surface expression of multiple markers, including CD45.2, CD11b, CD11c, F480, TCR, and Gr-1 in preparation for flow cytometric analysis.
ND = no detectable expression
Error bars indicate standard deviation form the mean
FIGURE 55

APC populations isolated from SV11 tumors and B6 brains following combinatorial immunotherapy

- CD45.2+/CD11c+ (total DC)
- CD45.2+/CD11c+CD11b- (BM-derived DC)
- CD45.2+/CD11c+/CD11b+ (BM-derived DC)
- CD11b+/F480+ (total macrophages)
- CD11b+/F480+/CD45+ (BM-derived macrophages)
- CD11b+/F480+/CD45- (brain-resident microglia)
- CD11c+TCR+ (CD11c+ T cells)
- GR-1+ (tumor-infiltrating granulocytes)

% total tumor (SV11) or brain (B6) cells

SV11  N.D.  N.D.  B6  N.D.  N.D.
and F480. In SV11 tumors total macrophages (CD11b+F480+) represented 12% of total cells. This macrophage population could be divided into 2 subsets according to hematopoietic origin (CD45). 7% of total CD11b+F480+ cells were bone marrow-derived (CD45+) and 5% represented brain-resident microglia (CD45-) (Figure 55). The macrophage population in B6 brains differed significantly from that observed in SV11 tumors. Total macrophages represented 18% of total brain cells and this population could be divided into 2% bone marrow-derived macrophages (CD45+) and 16% resident microglia (CD45-). In order to rule out the possibility that CD11c+ cells were activated T cells, tumor stromal preparations were co-stained for TCR and CD11c. This revealed that no TCR+CD11c+ cells could be detected in the brains of either mouse strain, confirming that CD11c+ populations truly represented DCs (Figure 55). Finally, some reports have shown that immune escape correlated with influx of Gr-1+ cells into the tumor stroma (Bronte et al., 1998; Gabrilovich et al., 2001; Kusmartsev et al., 2000). However, no Gr-1+ cells were detected in either SV11 tumors or B6 brains, ruling out this subset as a source of immune suppression (Figure 55). Thus, following combinatorial therapy, a unique tumor microenvironment in SV11 tumors is established, characterized by infiltration of bone marrow-derived CD11c+ DCs, a subset of which also express CD11b.

**Bone marrow-derived CD11c+ APCs that infiltrate SV11 choroid plexus tumors and CLNs following combinatorial immunotherapy express B7-H1 (PD-1 ligand)**

Since a distinct bone marrow-derived CD11c+ DC population was detected in SV11 tumors (Figure 55), the potential contribution of these myeloid DCs in the inhibition of SV11 tumor control following combinatorial therapy was investigated. CLN and tumors from 125 day-old SV11 mice and B6 mice that had received combinatorial therapy as well as CLN and brains from 105 day-old SV11 mice that received no treatment were assessed for the detection of CD11c+CD45.2+ bone marrow-derived DCs that expressed B7-H1 (Figure 56). In B6 mice, few B7-H1-expressing DC were observed in the brain or CLN (less than 2% of total cells), regardless of adoptive immunotherapy, although the frequency of B7-H1+ DC was somewhat elevated in CLN of B6 that had received treatment (4% of total cells) (Figure 56). In unmanipulated SV11 mice, CD45+CD11c+ cells that also expressed B7-H1 represented 8% of total cells in the CLN and 4% of total cells in tumors. The increased frequency of B7-H1+ DCs in the CLN might contribute to an immunosuppressive environment created within the LN immediately draining the tumor. In SV11 mice that had received combinatorial immunotherapy,
Bone Marrow-Derived CD11c+ APCs That Infiltrate SV11 Choroid Plexus Tumors and CLNs Following Combinatorial Immunotherapy Express B7-H1 (PD-1 ligand)

Stromal cells from choroid plexus tissue and CLN of SV11 mice and B6 mice (4 mice/group) that had received either no treatment or combinatorial therapy 40 days earlier were prepared by collagenase digestion. Isolated cells were co-stained for cell surface expression of CD45.2, CD11c, and B7-H1 in preparation for flow cytometric analysis. Error bars indicate standard deviation from the mean.
FIGURE 56

Bone Marrow-Derived CD11c+ APCs That Infiltrate SV11 Choroid Plexus Tumors and CLNs Express B7-H1 (PD-1 ligand)
a very different picture was observed. Bone marrow-derived B7-H1-expressing DCs represented 12% of total cells in the CLN and 18% of total cells in the tumor stroma (Figure 56). These results demonstrate that administration of combinatorial immunotherapy to tumor-bearing SV11 mice ultimately leads to the accumulation of B7-H1+ bone marrow-derived DCs in the tumor-draining LN and tumor site, suggesting the potential for tumor-infiltrating APC to provide the cognate PD-1 ligand responsible for inhibition of tumor-resident TCR-V cells.

Host derived T-CD8 that accumulate in SV11 tumors do not produce suppressor cytokines, but do express the inhibitory ligand B7-H1

A consistent observation made in all experiments involving TCR-V cell mediated combinatorial immunotherapy was the accumulation of host-derived (CD45.2+) Tag-V tetramer negative T-CD8 in SV11 brains (Figure 57A). There are several possible explanations for the presence of these endogenous T cells at the tumor site. They might represent bystander T lymphocytes that gained entry to the brain along-side initial TCR-V cell extravasation (Tough et al., 1996). Alternatively, they could be suppressor CD8+ T cells, which have been described in the literature. Such T cell populations may be generated from CD40 ligand-activated DCs and have been shown to infiltrate a variety of mouse and human tumors, including ovarian carcinomas, where they exert their suppressive function through production of IL-10 and TGF-β (Gilboa, 1999; Gilliet and Liu, 2002; Jarnicki et al., 2006; Wei et al., 2005; Zou, 2005, 2006).

In order to address the potential suppressor function of SV11 endogenous T-CD8 cells in choroid plexus tumors, SV11 spleens and brains were harvested at 40 days post combinatorial therapy and assessed for the production of activating cytokines (IFNγ and TNFα) and suppressor cytokines (IL-10 and IL-4) in response to non-specific stimulation via intracellular cytokine assay. Spleen and brain-isolated lymphocyte preparations were cultured for 5 hours with PMA/ionomycin + BFA, stained extracellularly for CD8 and CD45.2, and fixed and permeabilized prior to staining for intracellular accumulation of cytokines.

Analysis of activating cytokine production in the spleen demonstrated that the majority of CD8+Tag-V tetramer- cells produced TNFα (55% of total CD8+Tet- cells). A small population produced IFNγ only (5%) and an additional 20% produced both activating cytokines (Figure
FIGURE 57

Host Derived T-CD8 Cells That Accumulate in SV11 Tumors
Do Not Produce Suppressor Cytokines,
But Do Express the Inhibitory Ligand B7-H1

TCR-V cells were isolated from SV11 spleens and brains at 40 days post administration of combinatorial therapy.

A. A representative example of CD8+ cell populations isolated from SV11 brains according to specificity for Tetramer-V and expression of CD45.1. Histograms represent gated populations of CD8+ cells assessed for derivation according to CD45.1 expression (CD45.1+ = TCR-V donor derived; CD45.1- = SV11 host-derived).

B. In order to address the potential suppressor function of SV11 endogenous T-CD8 in choroid plexus tumors, SV11 spleens and brains (3 mice/group) were harvested at 40 days post combinatorial therapy, pooled, and assessed for the production of activating cytokines (IFNγ and TNFα) and suppressor cytokines (IL-10 and IL-4) in response to non-specific stimulation via intracellular cytokine assay. Spleen and brain-isolated lymphocyte preparations were cultured for 5 hours with phorbol myristate acetate (PMA) (2 ng/mL) and ionomycin (0.5 µM) + BFA, stained extracellularly for CD8 and CD45.2, and fixed and permeabilized prior to staining for intracellular accumulation of cytokines. Each bar graph indicates one sample well co-stained for either IFNγ and TNFα or IL-10 and IL-4, and indicate the % of CD8+ cells producing either one cytokine only or simultaneously producing both cytokines.

C. In order to assess the potential role for B7-H1 expression by T-CD8 cells in the inhibition of the TCR-V cell response to SV11 tumors, tumor-infiltrating cells from SV11 mice (4 mice/group) at 40 days post combinatorial therapy were stained for expression of CD8, Tetramer-V, CD45.1, and B7-H1. The data from the tumor of a representative SV11 mouse is shown. The dot plot on the left indicates the frequency of CD8+/Tet-V+ cells in the tumor. The dot plot on the right is gated on CD8+ cells to indicate B7-H1 expression by Tetramer-V+ and Tetramer-V- cells.
FIGURE 57

Host Derived T-CD8 Cells That Accumulate in SV11 Tumors Do Not Produce Suppressor Cytokines, But Do Express the Inhibitory Ligand B7-H1

A.

![Graph showing CD8+ T-cell distribution and Tetramer-V expression](image)

B.

![Bar charts showing cytokine production by tumor-infiltrating host-derived CD8+ lymphocytes](image)

C.

![Flow cytometry plots showing CD8+ T-cell gating](image)
Among T cells isolated from SV11 brains, the majority of CD8+Tag-V tetramer- cells produced IFNγ (30% of total CD8+Tet- cells); 5% produced TNFα; and 10% of total CD8+Tet-cells produced both TNFα and IFNγ (Figure 57B). Analysis of suppressor cytokine production found that in both spleen and brain lymphocyte preparations, negligible frequencies of CD8+Tet-cells above background (BFA only) produced IL-4 or IL-10 in response to non-specific stimulation (Figure 57B). These results demonstrate that the dramatic progressive accumulation of host-derived T-CD8 cells in SV11 tumors following combinatorial therapy do not appear to represent a suppressor T-CD8 cell population, leaving potential bystander infiltration of these endogenous T-CD8 cells as a feasible alternative.

Results from the current studies demonstrated that while B7-H1 as a potential ligand for TCR-V cell expression of PD-1 was not detected on SV11 choroid plexus tumors (Figure 54), B7-H1 expression was found on CD11c+ DCs (Figure 56). It is known that B7-H1 is constitutively expressed on T cells, B cells, macrophages, and DCs and is further up-regulated following activation (Ishida et al., 2002; Yamazaki et al., 2002). Thus, in the SV11 tumor model, the expression of B7-H1 on DC populations could be responsible for the inhibition of TCR-V cell responses toward SV11 tumors. However, reports from the literature suggest that high levels of B7-H1 may also be expressed by activated T-CD8 cells (Ishida et al., 2002; Yamazaki et al., 2002). Thus, it remained possible that tumor-infiltrating T cells expressed this ligand, and were thus contributory to PD-1 mediated inhibition of TCR-V cells.

In order to address another potential role for B7-H1 in the inhibition of the TCR-V cell response to SV11 tumors, the expression of B7-H1 on tumor-infiltrating CD8+ cells was assessed at 40 days post combinatorial therapy (Figure 57C). Assessment of B7-H1 expression on tumor-infiltrating CD8+ T cells revealed a surprising result. Both TCR-V (tetramer-V positive) and host-derived (tetramer-V negative) CD8+ cells expressed high levels of B7-H1 (Figure 57C). Further analysis confirmed that the cells in the Tetramer-V+ population were CD45.1+ (donor-derived) and cells in the Tetramer-V- population were CD45.1- (host-derived) (Figure 57A). Given that highly activated T cells gain preferential entry into the brain and aware of the potential for activating cytokine production to occur within the tumor, up-regulation of B7-H1 by both TCR-V cells and endogenous bystander T cells could potentially inhibit the proliferative and effector response of TCR-V cells in situ.
Blockade of B7-H1 (PD-1 ligand) restores *in vitro* proliferative capacity of TCR-V cells isolated from SV11 tumors

The expression of and signaling through the PD-1 inhibitory receptor has been shown to participate in cell cycle arrest and decreased cytotoxic function (Hirano et al., 2005; Iwai et al., 2002; Latchman et al., 2001). Additionally, antibody-mediated blockade of both PD-1 as well PD-1 ligands has been reported to restore T-CD8 proliferative and functional capacity both *in vitro* and *in vivo* (Barber et al., 2006; Blank et al., 2004; Curiel et al., 2003; He et al., 2004; Hirano et al., 2005; Iwai et al., 2002; Petrovas et al., 2006; Strome et al., 2003; Trautmann et al., 2006). In order to investigate the role of PD-1 in the induction of TCR-V cell anergy, TCR-V cells were isolated from SV11 brains and spleens at 40 days post-combinatorial therapy and cultured under various activating conditions in order to drive TCR-V cell proliferation. Both specific peptide stimulation and α-CD3 stimulation drive T cell activation via TCR engagement. Signaling via the TCR is believed to result in a variety of biochemical events that include a rise in intracellular free calcium and translocation of protein kinase C (PKC). These two signals also can be generated by calcium ionophores, such as ionomycin, and by activators of protein kinase C, such as phorbol myristate acetate (PMA) (Nau et al., 1988; Ohoka et al., 1997). Thus, TCR-V cells were CFSE-labeled and cultured *in vitro* for 6 days with feeder cells and (i.) IL-2 only; (ii.) IL-2 + Tag-V peptide (specific TCR-dependent stimulation); (iii.) IL-2 + PMA/ionomycin (non-specific TCR-independent stimulation); and (iv.) IL-2 + plate-bound α-CD3 (non-specific TCR-dependent stimulation. One half of the wells also included functional grade αB7-H1 mAb in order to block PD-1 ligation. An additional set of wells contained TCR-V cells isolated from SV11 spleens without inclusion of αB7-H1 mAb. *In vitro* cultures were assessed on day 6 to detect TCR-V cell proliferation as determined by CFSE dilution (*Figure 58*).

Just as previously observed (Figures 47 and 48), TCR-V cells isolated from SV11 spleens proliferated under all culture conditions containing IL-2, confirming that proliferative potential of peripheral TCR-V cells was maintained (*Figure 58 – bottom panel*). TCR-V cells isolated from SV11 tumors also reiterated earlier results (Figures 47 and 48), and did not proliferate in response to IL-2 only, Tag-V peptide, or α-CD3. However, stimulation with PMA/ionomycin was able to induce TCR-V cell proliferation (93% of total cells), indicating that TCR-V cell inhibition was exerted at the level of the T cell receptor (*Figure 58 – top panel*). By comparison, a very different result was observed in culture wells that also contained the αB7-H1 mAb. Inclusion of the PD-1 ligand blockade mAb rescued proliferation of brain-isolated TCR-V
FIGURE 58

Blockade of B7-H1 (PD-1 Ligand) Restores the in vitro Proliferative Capacity of TCR-V Cells Isolated From SV11 Tumors

TCR-V cells were isolated from 16 SV11 brains and spleens at 40 days post-combinatorial therapy, stained separately to assess consistent frequency of Tet-V+CD8+ cells. Cells from 13 mice were pooled, and cultured under various activating conditions in order to drive TCR-V cell proliferation. Tumor-isolated TCR-V cells were CFSE-labeled and cultured in vitro (1x10^5 cells/well) for 6 days with irradiated feeder cells (1x10^5 cell/well) and (i.) IL-2 only (10 U/mL); (ii.) IL-2 (10 U/mL) + 5µM Tag-V peptide (specific TCR-dependent stimulation); (iii.) IL-2 (10 U/mL) + phorbol myristate acetate (PMA) (2 ng/mL)/ionomycin (0.5 µM) (non-specific TCR-independent stimulation); and (iv.) IL-2 (10 U/mL) + plate-bound α-CD3 (10 ug/mL) (non-specific TCR-dependent stimulation). One half of the wells also included functional grade αB7-H1 mAb (10 ug/mL) in order to block PD-1 signaling. An additional set of wells contained TCR-V cells isolated from SV11 spleens (1x10^5 cells/well) + irradiated feeder cells (1x10^5 cell/well) under the same three stimulation conditions, but without inclusion of αB7-H1 mAb. In vitro cultures were assessed on day 6 to detect TCR-V cell proliferation as determined by CFSE dilution. Histogram markers indicate % of Tet-V+CD8+ cells that had undergone greater than 2 rounds of division.
FIGURE 58

Blockade of B7-H1 (PD-1 Ligand) Restores the in vitro Proliferative Capacity of TCR-V Cells Isolated From SV11 Tumors
cells under the culture conditions that included Tag-V peptide (95% proliferating) and \(\alpha\)-CD3 stimulation (98% proliferating), in addition to PMA/ionomycin activation (Figure 58 – middle panel). A small percentage of tumor-isolated TCR-V cells (35%) also proliferated in the well containing IL-2 only, which may be attributed to residual endogenous Tag or tumor cells in the culture. These results demonstrate that the PD-1/B7-H1 pathway does indeed contribute to the inhibition of tumor-isolated TCR-V cell activation and division, and that inhibition occurs at the level of the TCR-dependent signaling pathway. Given that B7-H1 is expressed by TCR-V cells, host-derived tumor-infiltrating CD8+ Tet-V- cells (Figure 57), and tumor-infiltrating CD11c+ DCs (Figure 56), the particular cell type for which blockade of B7-H1 rescues TCR-V cell proliferation remains unknown. However, these data suggest that SV11 tumor escape of TCR-V cell mediated immunotherapy may be thwarted through \textit{in vivo} blockade of PD-1/B7-H1 engagement.

**CONCLUSIONS**

From these studies it can be concluded that several factors participate in immune escape by SV11 tumors following combinatorial therapy, including alterations in both the T cells and within the tumor microenvironment. TCR-V cells that had infiltrated choroid plexus tumors manifested characteristics unique from TCR-V cells remaining in the periphery and demonstrated multiple examples of non-responsiveness. Brain-infiltrating TCR-V cells did not express memory markers including the IL-2\(\beta\) receptor and IL-7 receptor, and a distinct population had down-regulated Ly6C at the time of analysis, suggesting that persistent antigen provides constant activation for TCR-V cells accumulated in the tumor. In contrast, a significant proportion of TCR-V cells in SV11 tumors did express high levels of the inhibitory PD-1 receptor, as well as the cognate PD-1 ligand, B7-H1. The expression of PD-1 correlated with the functional anergy demonstrated by brain-infiltrating TCR-V cells, as these cells did not proliferate and did not down-regulate PD-1 \textit{ex vivo}, nor did they demonstrate cytotoxic function following isolation and re-transfer into an antigen-free environment. At late time points, although a fraction of tumor-infiltrating TCR-V cells demonstrated \textit{in vivo} proliferation and the capacity to produce IFN\(\gamma\), this did not correlate with ability to control tumor progression, as all mice eventually succumbed to tumor. Finally, a second round of combinatorial therapy did prolong SV11 survival, suggesting that tumors remained sensitive to TCR-V cell mediated
immunotherapy, and further supporting the conclusion that TCR-V cells are eventually rendered anergic. TCR-V cells in the periphery harbored very different characteristics. These cells proliferated ex vivo in response to Tag-V as well as to direct αCD3 stimulation. Peripheral TCR-V cells also expressed memory markers and demonstrated potent in vivo cytotoxic function.

The choroid plexus tumor microenvironment also demonstrated changes following immunotherapy. Tumors did not lose expression of MHC Class I molecules, nor did they express B7-H1. However, at 40 days post combinatorial therapy, multiple cell types infiltrated the tumor stroma, including cells of the myeloid lineage (DCs and macrophages) as well as non antigen-specific host-derived T-CD8 cells. Tumor-infiltrating CD11c+ DCs and host derived T-CD8 cells also expressed high levels of B7-H1. Rescue of the TCR-V cell in vitro proliferative response was achieved with both PMA/ionomycin stimulation as well as by blockade of B7-H1, strongly implicating the inhibitory PD-1/B7-H1 pathway in anergic rendering of TCR-V cells that persisted at the tumor site.

**DISCUSSION**

In the context of tumor immunology, tumor immunity or tumor immune tolerance refers to the success or failure of the immune system to reject a tumor. Not only do the tumor and its microenvironment drive the neoplastic process by fostering cancer cell proliferation and survival, the propagation of conditions that favor tolerance helps to protect the tumor from potential attack from the immune system. Clinically, the presence of high numbers of circulating tumor-antigen specific CD8+ T lymphocytes in cancer patients does not correlate with T cell infiltration into cancer tissues or tumor regression (Dudley et al., 2002a; Lee et al., 1999a; Rosenberg, 2001b). Leukocytes may inefficiently adhere to tumor endothelium, and histological analysis has shown that leukocytes are mainly present in the peritumoral stroma, but to a lesser degree in the tumor proper (Chen et al., 2003). Additionally, reduced leukocyte recruitment into the tumor parenchyma might be explained by aberrations in the tumor vasculature, which result from selective pressure on the tumor microenvironment to continuously remodel vessel architecture for sufficient growth. These alterations lead to disruption in the architectural and hierarchal organization of the vascular tree into arterioles, capillaries, and venules (Baluk et al., 2005; Jain, 2003; Munn, 2003). Such dysfunctional vessels may be incapable of expressing
adhesion molecules such as P-selectin, VCAM-1 and ICAM-1 (Griffioen et al., 1996; Nooijen et al., 1998; Piali et al., 1995; Tromp et al., 2000).

However, getting tumor specific T-CD8 cells into tumors is only the first hurdle, as the presence of infiltrating T cells in tumors does not always correlate with tumor regression, indicating that often tumor-infiltrating T cells are inefficient for controlling tumor growth (Blohm et al., 2002; Prevost-Blondel et al., 1998). In the SV11 mouse model of TCR-V cell mediated immunotherapy, the T cells are not hampered in their ability to traffic to the brain. Rather the combinatorial treatment promotes their early accumulation and persistence at the tumor site. These studies demonstrated that while a small fraction of tumor-infiltrating TCR-V cells were actively proliferating at late time points post adoptive transfer + α-CD40 triggering + well-timed immunization, the majority of these cells are rendered non-functional. The characteristics of the ultimate loss of anti-tumor control by TCR-V cells that had demonstrated a dramatic and prolonged response to combinatorial therapy was similar to the study performed by Nguyen et al. (Nguyen et al., 2002). In this report, peptide immunization against the tumor antigen followed 2 days later by administration of agonistic α-CD40 mAb elicited a potent antitumor CD8 T cell response that greatly enhanced lifespan of RIP-Tag2 mice with spontaneous pancreatic insulinomas. However, in this study, eventually tumor-bearing mice that had received combined immunotherapy experienced tumor relapse. It was suggested by the authors that therapeutic immunization with self-antigen must be performed in a potent, repetitive manner to effectively achieve control over tumor growth (Nguyen et al., 2002).

**Tumor-infiltrating APCs and Other Immunosuppressive Cells**

The tumor microenvironment is composed of tumor cells, endothelial cells, fibroblasts, different leukocyte subsets, and ECM proteins. An intricate interplay between the cellular and extracellular components determines success or failure of tumor progression (Hanahan and Weinberg, 2000; Liotta and Kohn, 2001). Macrophages may promote tumor growth, as they are attracted into tumors where they release proangiogenic cytokines (VEGF and FGF) (Coussens and Werb, 2002). Tregs may also counteract the activity of effector cells via secretion of IL-10 and TGF-β (Chiou et al., 2005; Yu et al., 2006; Yu et al., 2005; Zou, 2006). Increased numbers of Tregs have been observed in melanoma, NSLC lung cancer, ovarian cancer, pancreatic adenocarcinoma, and breast cancer (Wang et al., 2004; Woo et al., 2001; Zou, 2006).
The importance of the tumor microenvironment in post therapy monitoring has been demonstrated in multiple systems. Bone marrow derived APCs may also accumulate at the tumor site to induce tolerance (Bronte et al., 2001; Bronte et al., 1998; Danna et al., 2004; Gabrilovich, 2004). In a fibrosarcoma tumor model expressing LCMV gp33, tumors became infiltrated with CD11b+CD11c+ macrophage-like cells that were capable of cross-presenting the tumor-associated epitope to T cells (Blohm et al., 2002). Tumor-infiltrating T cells exhibited a highly activated phenotype, but lacked effector cell function, suggestive that tumor microenvironment is able to alter the functional activity of T cells infiltrating the tumor mass. Similarly, in the studies presented here, it was observed that multiple APC populations infiltrated SV11 tumors following combinatorial therapy, and that the CD11c+ population expressed the B7-H1 inhibitory molecule.

In an EL4 transplantable tumor model adoptively transferred with OT-1 TCR-Tg cells it was shown that a population of CD11b+GR-1+ immature myeloid cells accumulated in large numbers in the spleen, LN, and tumor tissues of tumor-bearing mice, and were capable of inducing tolerance (Bronte et al., 1998). These cells took up whole soluble protein in vivo, processed and presented it to induce T cell anergy and apoptosis. Immunosuppressive CD11b+ myeloid cells have also shown to correlate with failed attempts by T-CD8 cells to control ocular tumor growth in the immune-privileged anterior chamber (McKenna and Kapp, 2006). Additional tumor-infiltrating APC-associated inhibitory mechanisms within the tumor microenvironment include macrophages that express the newly identified member of the inhibitory B7 family – B7-H4 (Zou, 2005), tumor environmental IDO+ myeloid DCs (Munn et al., 2002) CXCR4+ plasmacytoid DCs (Salio et al., 2003; Zou et al., 2001), VEGF-responsive tumor vascular DCs (Conejo-Garcia et al., 2004), and production of TGF-β, reactive oxygen species, and peroxynitrites by CD11b+GR-1+ myeloid suppressor cells (Kusmartsev et al., 2000; Mazzoni et al., 2002). Multiple populations of myeloid cells were seen to infiltrate SV11 tumors, including CD11b+ and F4-80+ cells in addition to B7-H1+CD11c+ population. The presence of TGF-β has been detected in progressive SV11 tumors following therapy involving IL-12 adminstration (Roy et al., 2000). Thus, while brain-infiltrating Gr-1+ cells were not detected in our studies, the remaining APC populations represent potential candidates for the production of immunosuppressive factors including TGFβ and reactive oxygen species.

Regulatory T-CD8 cells may also be recruited into tumors as observed in a CT26 colon carcinoma model, in which macrophages, DCs and T-CD8 cells that infiltrated tumors secreted
IL-10 and TGFβ (Jarnicki et al., 2006). In the Her-2/neu mouse model, intratumoral immunotherapy with IL-12 and GM-CSF induced transient regression. However, T suppressor cells homed to tumors as rapidly as tumor specific activated CD8+ T cells (Nair et al., 2006). While the presence of CD4+Foxp3+ Tregs was not addressed in the current studies, infiltrating CD8+ Tregs as a possible immunosuppressive mechanism in SV11 tumors was ruled out.

**PD-1/B7-H1 Mediated Inhibition**

The studies presented here illustrate the potential for the PD-1/B7-H1 inhibitory cascade to impede T-CD8 cell mediated anti-tumor responses (Blank and Mackensen, 2007). The combined high-level expression of PD-1 by tumor–infiltrating TCR-V cells and B7-H1 by both tumor-infiltrating T cells and DCs suggested the possible role for this pathway in the induction of TCR-V cell non-responsiveness. This proposal was strengthened by the ability for blockade of B7-H1 to rescue TCR-V cell proliferation *in vitro*. B7-DC, the other ligand for PD-1, was not investigated in these studies and thus cannot be ruled out as additionally contributory to TCR-V cell inhibition. B7-H1 expression differs from B7-DC in that B7-H1 is expressed on activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells. B7-DC is expressed constitutively in the liver, lung, and spleen, placental endothelium and medullary thymic epithelial cells, and is inducibly expressed on DCs and macrophages (Ishida et al., 2002; Latchman et al., 2001; Tseng et al., 2001; Yamazaki et al., 2002). While both ligands have shown expression on tumor cells procured from human patients, including lung, breast, ovary, kidney, and brain (Curiel et al., 2003; Dong et al., 2002; Konishi et al., 2004; Ohigashi et al., 2005; Okazaki and Honjo, 2006; Strome et al., 2003; Thompson et al., 2004; Wintterle et al., 2003), B7-H1 is highly expressed on most carcinomas and minimally expressed on adjacent normal tissue. Thus, in these studies, attention was focused on the potential role for B7-H1 in the attenuation of the TCR-V cell-mediated anti-tumor responses in SV11 mice.

Several observations have been made in separate experimental systems in attempts to explain mechanisms responsible for PD-1/B7-H1 mediated inhibition. In some systems, B7-H1 blocks cell cycle progression in activated tumor specific T cells, but does not promote apoptosis (Latchman et al., 2001). In contrast, other studies have provided evidence that B7-H1 leads to activated T cell apoptosis, rescuable by blocking with B7-H1 mAb (Dong et al., 2002). It has been shown that blocking B7-H1 with mAb stimulated proliferation of anergic human T-CD8 cells that had been induced by IL-10 treated DCs (Selenko-Gebauer et al., 2003). Similarly,
Trautmann et al. (Trautmann et al., 2006) observed that in chronic HIV infection, up-regulation of PD-1 on HIV-specific T-CD8 cells isolated from human patients correlated with a depressed proliferation and effector cytokine production. However, mAb blockade of B7-H1 restored survival, proliferation, and cytotoxic function of virus-specific T-CD8 cells in vitro. In a mouse model of conditional epitope expression, Probst et al. (Probst et al., 2005) reported that B7-H1 on resting DCs was responsible for the induction of tolerance, which could be subverted with either adoptive transfer of PD-1 -/- T cells or administration of anti-CD40 mAb (Probst et al., 2005). Recently, Martin-Orozco et al. (Martin-Orozco et al., 2006) found a role for PD-1/B7-H1 interactions in the induction of OT-1 cell tolerance to pancreatic self-antigen in RIP-OVA mice. However, OT-1 cell proliferation and effector function were restored following in vivo receptor blockade with either mAbs to PD-1 or B7-H1 (Martin-Orozco et al., 2006).

One of the first studies to exemplify the role of the PD-1/B7-H1 pathway in tumor escape was conducted by Iwai et al. (Iwai et al., 2002) who showed that transgenic expression of B7-H1 in P815 tumor cells rendered tumors less susceptible to lysis by antigen specific-cytotoxic T cells in vitro, and markedly enhanced their tumorigenesis and invasiveness in vivo compared to tumors that did not express B7-H1. Both of these effects were reversible with anti-B7-H1 mAb. Tumor environmental B7-H1 and PD-1 appear to play critical roles in the interaction between T cells, tumor cells, and APCs. These interactions present novel mechanisms by which tumors evade the immune system and pose as possible candidates for loss of TCR-V cell responsiveness to SV11 tumors (Curiel et al., 2003; Zou, 2005). The SV11 data shows that B7-H1 is expressed on several cell types in the choroid plexus tumor environment, including DCs and T cells. Tumor factors within the local tumor microenvironment may affect the functional capacity of DCs to activate T cell tumor antigen-specific immunity, by triggering B7-H1 expression on DCs in tumors and DLN (Curiel et al., 2003). Blocking B7-H1 has been shown to enhance myeloid DC mediated T cell activation (Brown et al., 2003; Curiel et al., 2003) and restore anti-tumor responses of T cells (Blank et al., 2004; Dong et al., 2002; Iwai et al., 2002). A very recent study found B7-H1 to be constitutively expressed on human renal cell carcinoma cell lines and up-regulated on human melanoma cell lines upon exposure to IFNγ, but not on normal tissues (Blank et al., 2006). Additionally, PD-1 was expressed at higher levels on tumor-infiltrating lymphocytes than on peripheral blood lymphocytes from melanoma patients following specific antigen stimulation. While antigen specific T-CD8 cells did not respond to stimulation with antigen-pulsed DCs, B7-H1 blockade increased the production of cytokines and cytolytic activity (Blank et al., 2006).
Hirano et al. addressed the role of B7-H1 in tumor resistance to T-CD8 cell-mediated immunotherapy in mice and in vitro cytotoxicity in several different tumor types, including P815 mastocytoma, B16 melanoma, and 4T1 breast tumors. It was found that high expression of B7-H1 on tumors conferred resistance to lysis and led to progressive disease in mice – effects that were abrogated upon *in vivo* blockade with anti-PD1 or anti-B7-H1 mAbs. The mechanism in this case was not attributed to induction of T cell anergy, but due to a “molecular shield” established following PD-1/B7-H1 engagement, shielding the tumor from lysis. It was hypothesized that B7-H1 integrates within the immunological synapse when tumor cells engage activated T cells such that TCR-MHC interaction is disrupted. In support of this proposal, another report indicated that PD-1 co-localization with TCR is required for its inhibitory function, which suggested that B7-H1 on tumor cells could bind directly to PD-1 in order to prevent TCR-mediated recognition or signaling (Chemnitz et al., 2004).

Thus PD-1/B7-H1 mediated inhibition may also explain the correlation made between cancer patents with progressive disease and tumor cell expression of B7-H1 (Ohigashi et al., 2005; Thompson et al., 2004). Curiel et al. (Curiel et al., 2003) found that B7-H1 was expressed on myeloid DCs isolated from tissues and DLNs of human ovarian carcinomas. Blockade of B7-H1 restored activation and IFNγ production of allogenic T cells against the tumors. Furthermore, T cells conditioned with an antagonistic mAb to B7-H1 demonstrated elevated ability to inhibit growth of human ovarian carcinomas in NOD-SCID mice (Curiel et al., 2003). While B7-H1 is not expressed on SV11 choroid plexus tumors, the interaction between PD-1 on TCR-V cells and B7-H1 on other T cells or CD11c+ DCs in the tumor microenvironment plausibly lead to similar consequences, preventing the TCR-V cells from initiating effector signaling cascades post tumor recognition. Indeed the data indicate that the PD-1 mediated inhibition of TCR-V cells occurs at the level of the TCR – as neither Tag-V peptide stimulation nor α-CD3 stimulation could drive tumor-isolated TCR-V cells to proliferate *in vitro*. However, stimulation with PMA/ionomycin, which circumvents TCR signaling and directly activates PKC and induces CA++ flux was capable of inducing proliferation of these cells, even in the absence of B7-H1 blockade. This proposal could be tested by assaying for phosphorylated intermediates that precede PKC in the TCR signal cascade, such as phosho-ZAP-70, in an attempt to address the location of the blockade.
Interestingly, IFNγ strongly stimulates B7-H1 expression on APCs, implicating the potential for functional effector T cells in the tumor to contribute to APC-mediated tolerance induction (Dong and Chen, 2003; Dong et al., 2002). This cytokine may induce the observed up-regulation of MHC class I on tumors, while simultaneously inducing up-regulation of PD-1 ligands on other cells in the tumor stroma. In some tumor settings, a significant fraction of tumor-associated T cells are T regulatory cells (Curiel et al., 2004), which also express PD-1 ligands (Chen, 2004). Tregs can suppress IL-12 production by myeloid DCs, thereby reducing immunogenicity. It was found that both TCR-V cells and non antigen-specific host T cells expressed B7-H1 — thus representing alternative candidates for induction of the PD-1/B7-H1 inhibitory cascade. While B7-H1 blockade restored function in apparently anergic TCR-V cells, the B7-H1-expressing cell directly responsible for inhibiting TCR-V cells remains unknown. These results suggest that the expression of B7-H1 by either TCR-V cells, host-derived bystander T-CD8 cells, or CD11c+ DCs signifies a potent mechanism for the induction of TCR-V cell anergy, potentially permitting SV11 tumors to escape TCR-V immunosurveillance. Thus, the data from the current studies agree with many of the recently published reports and also implies that in vivo blockade of PD-1/B7-H1 interactions may provide a promising strategy for specific tumor immunotherapy, suggesting the potential for in vivo PD-1 blockade to restore TCR-V cell function correlative with a further delay in SV11 disease progression. Such therapeutic potential has already been recognized clinically, and although no data is yet available, Phase I clinical trials have been initiated in cancer patients utilizing a fully human anti-PD-1 mAb (Melero et al., 2007).

**Loss of Tumor Antigen Expression**

Tumors also have the potential to essentially hide from T cell recognition as a mechanism of escape from immune-surveillance. Although tumor cells may retain expression of the targeted antigen, alterations in the antigen-processing machinery including mutations in or loss of expression of MHC class I molecules, β2-microglobulin, and molecules associated with TAP or the immunoproteosome may result in failure to adequately present immunogenic epitopes to tumor-specific T-CD8 cells (Ferrone and Marincola, 1995; Marincola et al., 2000; Restifo et al., 1993; Rosenberg, 2001b; Vitale et al., 1998; Wang et al., 1996; Zitvogel et al., 2006). Tumors may also immuno-select not to express particular antigens, and such escape variants have demonstrated resistance to T cell mediated cytotoxicity (Saleh et al., 2001; Shankaran et al., 2001; Urban et al., 1982), and have been identified as a possible mechanisms allowing for tumors to escape immunotherapy in relapsing patients (Jager et al., 1997;
Ohnmacht et al., 2001; Ward et al., 1990; Yee et al., 2002). In the investigations presented here, the specific loss of Tag-V expression by choroid plexus tumors was not assessed. However, the ability for a second bolus of TCR-V cell adoptive therapy to significantly prolong SV11 survival suggests that the epitope was indeed available for TCR-V cell recognition, thereby permitting SV11 tumor susceptibility to therapy. It was also found that not only do SV11 tumors not lose expression of H-2D\(^b\) following combinatorial immunotherapy, MHC I was expressed at even higher levels in these mice, eliminating such mechanisms as means of tumor escape in the SV11 system.

**Induction of Apoptosis**

The inability to cure established tumors has been suggested to result from impaired immune response as a result of anti-tumor T cell death (Finke et al., 1999), and expression of FasL by tumors may be the basis for such induction of apoptosis and tolerance (Hahne et al., 1996). In a transplantable tumor model, tumor-infiltrating T cells were characterized for activation profile, cell cycle status, apoptosis, proliferation, and cytokine secretion. These cells expressed activation markers and contained IL-2 and IFN\(\gamma\) mRNA, but did not proliferate or secrete these cytokines, nor were they apoptotic in situ. Upon isolation and activation, cells did secrete cytokine and underwent AICD mediated by Fas. Thus, in this system, the T cells expressed both Fas and FasL, were arrested in G phase, did not secrete cytokine, and upon activation died by AICD (Radoja et al., 2001). In the SV11 model, TCR-V cells did not appear to apoptose in vivo, as significant numbers of cells persisted following combinatorial therapy. However, these cells appeared to have been rendered non-responsive. It remains possible that activated TCR-V cells do eventually die after several rounds of proliferation, and might be surmised by the observation that TCR-V cells isolated at 20 days post therapy proliferated ex vivo, but never accumulated in the CFSE negative channel. Thus, following combinatorial therapy, TCR-V cells may initially undergo apoptosis, followed later by anergic rendering of the remaining cells at the tumor site. Given the prominent expression of Fas and FasL in the CNS (Choi and Benveniste, 2004), the role of these molecules in modulating the TCR-V cell response to SV11 tumors remains undetermined.

**Mechanisms of T-CD8 Cell Functionality**

A fraction of tumor-infiltrating TCR-V cells were found to produce IFN\(\gamma\) upon Tag-V peptide stimulation. However, despite this apparent functional capacity in some of the cells at the tumor site, SV11 tumors progressed. Other reports have shown that IFN\(\gamma\) secretion, a factor
commonly used to monitor patient immunity does not necessarily correlate with the efficacy of tumor kill, and that monitoring of other functional parameters, such as granzyme/perforin secretion patterns of intra-tumoral or TDLN T cells may provide a better correlate (Nair et al., 2006). At this point, however, the potential of TCR-V cells to produce these cytotoxic molecules remains unexplored.

In conclusion, the assessment of the SV11 tumor microenvironment including persistent TCR-V cells, infiltrating APCs and host-derived bystander T cells, and direct tumor analysis revealed several mechanisms by which SV11 tumors progress after an initial period of tumor control following combinatorial therapy. Several of these mechanisms may cooperate to achieve immune escape, such as up-regulation of PD-1 on tumor-infiltrating TCR-V cells and the expression of B7-H1 on tumor-infiltrating APCs. However, despite the induction of non-responsiveness in TCR-V cells, anergy was reversible upon blockade of the PD-1 inhibitory receptor. Furthermore, tumors did not appear to lose presentation of the Tag-V epitope, as they were receptive to a second treatment with functional TCR-V cells, permitting significantly greater control over SV11 survival. These data demonstrate that while T-CD8 cells specific for immunorecessive tumor epitopes are also subject to the same forms of tolerance as T cells specific for dominant epitopes, such epitopes represent realistic targets against which immunotherapeutic strategies may be manipulated for prolonged successful anti-tumor control.
CHAPTER VII

BROAD IMPLICATIONS AND SIGNIFICANCE

Despite decades of intense research, cancer remains an enigmatic challenge for the immune system. One of the greatest obstacles in patients with tumors that express self-antigen epitopes is the paucity of T-CD8 cells that harbor potent tumor-reactivity. The studies presented in this manuscript have attempted to closely mirror the human cancer scenario using the SV11 mouse model, and report a quantitative and qualitative study monitoring the fate of T-CD8 cells specific for an immunorecessive tumor epitope in the context of a spontaneously arising tumor. The finding that TCR-V cells recognize endogenous Tag and respond to immunization in a manner that leads to tumor infiltration and prolonged survival of SV11 mice provides a foundation for continued investigations that target T cells specific for similar subdominant or immunorecessive epitopes. Additionally these studies explored immunotherapeutic strategies that augmented and sustained TCR-V cell responses in SV11 mice, as well as escape mechanisms with the potential to thwart successful anti-tumor immunity, thus providing pre-clinical clues toward the development of immunotherapeutic approaches for the treatment of cancer.

IMMUNORECESSIVE TUMOR EPITOPES

A prime goal in cancer immunotherapy is to break immunological tolerance to non-mutated tumor antigens and induce an effective anti-cancer immune response (Levitsky, 2000; Melief et al., 2000a; Smyth et al., 2001). As many normal tumor self-antigens are tolerizing, posing an obstacle to generation of anti-tumor activity, breaking tolerance may require distinct signals in different models. One of the factors in favor of these studies was the decision to target the immunorecessive Tag epitope-V. T-CD8 cells specific for this epitope probably most closely mimic T cells found in the residual repertoire of cancer patients, as more dominant epitopes are increasingly subject to tolerizing deletion. Thus, it can be proposed that many of the observations made in respect to the response of TCR-V cells toward SV11 tumors will translate to similar results achievable in the clinic.
One of the limitations often associated with focusing on such immunorecessive epitopes lies in their weak potency in response to specific antigen, as was observed in the initial studies indicating that upon adoptive transfer into SV11 mice, high-avidity TCR-V cells were incompletely activated and exerted no therapeutic effect. However, it became clear that these cells harbored dramatic therapeutic potential, evidenced by their capacity to respond to several immunotherapeutic modalities targeted at enhancing an initially weak response. It can be proposed that identification of similar epitopes in various forms of human cancer will reveal novel approaches for targeting tumors clinically that demonstrate comparable responsiveness in tumor-specific T cells, enhanced resistance to tolerance, and considerable efficacy.

ADOPTIVE IMMUNOTHERAPY AND CURRENT CLINICAL STATUS

The current status of immunotherapeutic approaches to multiple forms of cancer in the clinic, including non-Hodgkin’s Lymphoma, prostate cancer, renal cell carcinoma, malignant melanoma, colorectal cancer, and non-small cell lung cancer is primarily limited to variations on vaccination with tumor antigen-pulsed DCs. Some studies have also addressed the potential for vaccine-primed cells isolated from tumors or TDLNs or to exhibit in vivo anti-tumor activity. Generally speaking, combined evidence indicates that only adoptive cellular transfer approaches have yielded true demonstrable clinical success. Additionally, only one solid cancer type – melanoma - has yielded significant clinical results following tumor immunotherapy. Melanomas are among the most immunogenic of tumors and have repeatedly been shown to induce natural tumor-specific T cells in patients. Furthermore, these T cells are frequently found to infiltrate tumors. As melanomas are relatively easy to access surgically, excised tumors represent a source of tumor-specific T cells that can be studied ex vivo, expanded in culture, and potentially re-infused into patients. Additionally, much effort has been targeted toward revealing tumor associated melanoma antigens, and identifying specific epitopes for these antigens. In contrast to the lack of consistent clinical effectiveness of current cancer vaccines for melanoma and other cancers, the adoptive transfer of melanoma antigen-reactive lymphocytes under specific conditions, specifically nonmyeloablative lymphodepletion and IL-2 administration has demonstrated objective clinical responses in 50% of treated patients (Dudley et al., 2002a; Dudley et al., 2005).
Similar to the preliminary investigations of melanoma and other tumor varieties, the studies described here also faced initial challenges, primarily centered on the lack of TCR-V cell persistence following multiple approaches aimed at augmenting the TCR-V cell response in SV11 mice. However, the success of the combinatorial therapy in enhancing the persistence of TCR-V cells demonstrated that this observed effect correlated with a significant degree of anti-tumor control. Unfortunately, although these cells were maintained long-term, they lost the ability to respond to stimulation due to anergic rendering. Importantly, a second transfer of TCR-V cells demonstrated the capacity to extend survival another 40 days in spite of the presence of tumor. Rosenberg and colleagues have proposed that two of the critical parameters responsible for successful cancer immunotherapy include persistence of tumor-specific cells and telomere length of the cells – directly indicative of their proliferative history and indirectly related to their functionality. In the SV11 system, an assessment of the telomere length of TCR-V cells from the initial adoptive transfer compared with telomere lengths of the cells from the second transfer, may provide additional clues explaining the diverse capacities of the T cells to affect the tumor and disease progression. Such findings would suggest that in human cancer, patients may also respond to strategically-timed repeat bolus infusions of tumor-specific T cells, provided the tumors remain sensitive to immunotherapy.

The somewhat bittersweet juxtaposition of minimally significant clinical progress in other cancer types as compared with the recently successful outcomes in melanoma regimens raises the bar for basic scientists and clinicians alike. We are faced with the grim reality that every cancer is unique and will necessitate arduous study to determine avenues of therapeutic approaches optimal for targeting the particular cancer type in patients. Nonetheless, we should be encouraged by the achievements of the melanoma studies, and allow factors in those and other systems shown to facilitate observed successes to guide focused new investigations in other tumor systems.

TIMING OF VACCINANTION

The data from some of the studies presented here offer intriguing observations, potentially calling for re-assessment of current therapeutic regimens. In particular, the importance of carefully timed immunization of tumor-bearing hosts following adoptive cellular
therapy was appreciated. Based on the observations in these investigations, the following model is proposed (Figure 59):

For a tumor that has been growing insidiously in a patient, antigen has presumably been draining to TDLNs and may have already generated an immunosuppressive environment within the sentinel node, including accumulations of tolerizing APCs and T regulatory cells. Nonetheless, antigen is available and adoptively transferred T cells may be activated soon after transfer. For an immunorecessive epitope that harbors many of the characteristics exhibited by Tag-V, including unstable peptide-MHC complexes and poor cross-presentation potential, immunization on the day of adoptive transfer may ineffectively activate T cells already engaged by APCs presenting endogenous tumor antigen. Thus by the time T cells have recovered from this refractory period and regain the ability to respond to vaccination, the antigen derived from immunization may have already been cleared. Thus, adoptively transferred cells, instead of exerting therapeutic efficacy, have potentially been tolerized, and are either deleted or rendered non-responsive. In these studies, a one-week rest in tumor-bearing SV11 mice provided the time necessary for TCR-V cells to recover from endogenous antigen stimulation and respond to antigen derived from the Tag-V targeted cellular vaccination – which essentially served as a booster immunization. The dramatically different response of the transferred TCR-V cells to the day 7 immunization vs. the day 0 immunization, namely expansion, tumor-infiltration, and control of tumor progression – indicates the critical nature of properly timed immunization following adoptive transfer into a tumor-bearing host, and implicates the need for current vaccination regimens to undergo similar evaluation in order to determine optimal windows and schedules of immunization in patients.

CD40-MEDIATED ENHANCEMENT OF TUMOR IMMUNITY

The ability to mimic the characteristics of mature DCs using co-stimulatory molecules, cytokines, or agonistic mAbs such as α-CD40 in order to stimulate tumor has been shown to enhance T-CD8 cell expansion and survival of adoptively transferred T cells in vitro and in vivo. These studies further support findings made in other systems, and demonstrate the potency of α-CD40 conditioning on activation of tumor-specific T cells against endogenous antigen. However, to our knowledge, this is the first report of the dramatic effect of α-CD40 to augment the anti-tumor function of T-CD8 cells specific for an immunorecessive tumor epitope.
Timing of immunization plays a critical role in the development of immunotherapeutic strategies involving adoptive cellular transfer into tumor-bearing hosts. For example, in the SV11 mouse model:

A. When TCR-V cells are adoptively transferred into SV11 mice, tumor antigen that has drained from the choroid plexus is already available for immediate activation in the CLN.

B. While TCR-V cells are engaged by self-antigen, they may be refractory to Tag derived from immunization, especially when immunization is delivered on the same day as cellular transfer. By the time TCR-V cells have undergone contraction and regain the ability to respond to immunization, antigen from immunization delivered on day 0 may have already been cleared.

C. However, it remains feasible that TCR-V cells are capable of responding to exogenous antigen when immunization is delivered at later time points following adoptive transfer.
FIGURE 59

A. SV11 mouse expresses Tag in CLN

B. - TCR-V recognition of endogenous Tag
   - Immediate activation in CLN
   - TCR-V cells refractory to immunization

C. Following contraction TCR-V cells are able to respond to exogenous immunization
The promising data generated from these and other studies warrant evaluation of the use of similar therapies in the clinic. Before such a therapy can be initiated, critical factors must be considered. The efficacy of agonistic $\alpha$-CD40-reactive mAbs for clinical application however, is bound by pharmacokinetic constraints especially in solid tumors. Additionally, the likelihood of inducing human anti-mouse neutralizing antibodies poses limitations for therapeutic efficacy. Finally, the potential repercussions of systemic treatment with either $\alpha$-CD40 mAb or recombinant CD154 protein, including toxicity from proinflammatory cytokine production by CD40-activated endothelial cells must be appreciated (Grewal and Flavell, 1998; Schonbeck and Libby, 2001; Singh et al., 2001). A dramatic example of the potential serious adverse effects of administration of an immunostimulatory mAb was observed in the TeGenero placebo-controlled phase I study of the safety of TGN1412 - a superagonisitc anti-CD28 mAb - in which immediately following infusion, healthy participants developed systemic inflammation and multi-organ failure due to induction of a cytokine storm (Kenter and Cohen, 2006; Suntharalingam et al., 2006).

In order to address some of these safety parameters, a Phase I clinical trial was conducted in 2001, in which patients with solid tumors or intermediate-high grade non-Hodgkin Lymphoma were evaluated following treatment with subcutaneously administered recombinant CD154 (rhuCD40L) (Vonderheide et al., 2001). The maximal tolerated dose and serum half-life was determined and patients were evaluated for tumor responses and adverse effects. The results found 38% patients with stable disease, 6% with observed responses, and one patient who demonstrated complete remission. Furthermore, the only adverse effects of therapy consisted of transient elevations in hepatic transaminases. Similar results have been observed in clinical trials that administered humanized anti-CD40 mAb to patients at dosages comparable to those used in the current SV11 studies as well as in other mouse models (Advani, 2005; Gladue, 2006; Hunter et al., 2007; Vonderheide, 2006; Vonderheide et al., 2007). Based on the data from these studies aimed at assessing the safety of anti-CD40 mAb-mediated treatments in the clinic, the inclusion of this modality in human tumor-targeted immunotherapeutic protocols holds great promise (Geldart, 2005; Tong and Stone, 2003; Vonderheide, 2007).
COMBINATORIAL IMMUNOTHERAPY

T cell survival poses a major hurdle that impedes the success of adoptive T cell therapy in the clinic. It was only recently that T cell persistence was shown to correlate with observed clinical responses (Robbins et al., 2004). The studies presented here demonstrate the ability to synergistically enhance an anti-tumor response targeted against an epitope that initially showed poor potential for effective tumor control. The success in the fused targeting of adoptively transferred T cells against both endogenous and exogenous forms of the tumor antigen offers multiple possibilities for success in similar approaches toward other tumor types. In addition to α-CD40 conditioning, mechanisms of stimulation against endogenous antigen include administration of TLR agonists, such as unmethylated CpG motifs. With respect to immunization, the SV11 studies provide support for the use of cellular-based vaccination, as this route of immunization has the potential to yield significantly greater responses than peptide alone or peptide-pulsed DCs, since tumor-antigen or tumor-antigen variant expressing cells provide sources of both cross- and directly-presented antigen.

For specific targeting of immunorecessive epitopes in the clinic, the generation of cells derived from either DCs or HSCs transfected with tumor antigen variants may promote activation of immunorecessive tumor epitope specific T cells unhindered by T cells specific for immunodominant epitopes (Banchereau and Palucka, 2005; Cui et al., 2003; Furumoto et al., 2004; Okano et al., 2005). One advantage of combination therapy lies in the option to introduce new modalities into the immunotherapeutic regimen, potentially targeting another pathway, either via enhancement of immune-activating conditions or dampening immune-suppressive conditions. For example, in the SV11 model, the accumulation of non-responsive TCR-V cells in SV11 tumors suggests that these cells are merely taking up space without contributing to tumor control. Thus, lymphodepleting irradiation at this point in therapy could provide benefit in several ways – (i.) potential reduction in tumor burden; (ii.) release of antigen from tumor by apoptotic cells; (iii.) removal of anergic T cells in the tumor; (iv.) creation of a lymphopaenic environment hospitable for the expansion of freshly transferred T cells; and (v.) changes in tumor vasculature promoting infiltration of newly transferred cells into the tumor bed.
IN VIVO TARGETING OF INHIBITORY PATHWAYS

These studies contribute to the recent field of data that has demonstrated an appreciation for the role of the PD-1/B7-H1 pathway in subversion of immune mediated anti-tumor responses. The exploitation of a pathway, likely intended for maintaining immune status quo and preventing autoimmune disease, by multiple tumor types to escape immune surveillance suggests promise for therapies aimed at inhibiting PD-1/PD-1 ligand interactions in multiple cancers. Although the ability to target brain tumors in vivo with mAbs to PD-1 and PD-1 ligands presents a challenge for penetration of the BBB, such systemic therapy may prove effective for other more easily accessible solid tumors. As with any treatment targeted at inhibiting an otherwise normal pathway, adverse effects of such therapy would need to be closely monitored for potential development of autoimmune symptoms. Nonetheless, the data generated in animal systems in vivo and with human cells ex vivo provides convincing evidence for the therapeutic efficacy of blocking such a significant inhibitory cascade.

OVERCOMING TOLERANCE IN IMMUNE-PRIVILEGED SITES

A realistic challenge posed by the SV11 model is the anatomical location of choroid plexus tumors. A disadvantage to immune privilege in the CNS is that brain tumors may grow unhampered leading to severe morbidity and mortality. The tumor may grow quietly and insidiously without patient recognition until severe symptoms result from a large tumor mass. Thus, amongst solid tumors, brain tumors such as choroid plexus papillomas require that potent and persistent anti-tumor mechanisms be imposed by the immune system in order to eliminate or even control further progression over neoplastic masses of considerable size. Treatment of some brain tumors also faces the challenge of access to the tumor. Many CNS tumors are situated in strategically difficult or impossible locations for surgical access. Thus, the tumor cannot be debulked, and clinicians must resort to the current remaining alternative therapies, including protocols that involve irradiation and chemotherapy.

Unfortunately, both irradiation and chemotherapeutic approaches often indiscriminately destroy normal tissues, and patients must contend with potentially permanent neurological changes or severe damage induced from side effects associated with such treatments. However, as tumors are frequently highly vascularized, peripheral blood cells may gain close
access to the tumor as they course through the circulation. Thus, provided a tumor specific T cell has been activated against the tumor, there is potential for such cells to extravasate and traffic to a surgically inaccessible site. Herein lies the challenge for tumor immunologists – to optimize the capacity for expansion, migration, and accumulation of functional tumor-reactive T cells within the tumor. Such an accomplishment also poses complex challenges, as the presence of tumor-specific T cells in the periphery does not necessarily correlate with numbers of T cells that have infiltrated the tumor. Thus, it has been proposed that breaking the tumor barrier, thus allowing T cell recruitment to the peripheral tissue in which they are needed, presents a critical hurdle limiting the success of anti-tumor immunotherapy. In response to this challenge, the results from these studies demonstrate that immunotherapeutic approaches such as immunization or activation against endogenous Tag with α-CD40 conditioning, endows immunorecessive epitope-specific TCR-V cells with the ability to traffic to and infiltrate choroid plexus tumors, where they persist long-term and exert significant control over disease progression.

**SUMMARY**

Just as tumors have the potential to mutate as a means of promoting their own survival and metastasis, so must immunotherapeutic approaches adapt in order to accommodate such changes and maximize anti-tumor efficacy. The findings presented here implicate critical checkpoints during tumor progression at which therapeutic intervention is necessary. These include: (i.) optimizing initial enhancement of the priming event against endogenous tumor antigen, (ii.) providing appropriately-timed immunization to prolong the anti-tumor response, and (iii.) monitoring disease at late time points to overcome negative regulation of persisting cells (Figure 60). These studies demonstrate the potential for such strategically-executed immunotherapeutic manipulations to dramatically augment a relatively weak anti-tumor response of immunorecessive epitope-specific T-CD8 cells in the face of advanced tumors, and imply that similar combinatorial therapeutic approaches toward cancer patients could yield promising results in clinical practice.

It is hoped that these investigations have shed more light on understanding the intricacies associated with targeting tumors through the relatively unexplored realm of immunorecessive tumor epitopes. These studies take their place in the ongoing quest for revealing strategies with promise for clinical translational efficacy, and are offered as an
additional piece toward understanding the baffling puzzle of how the immune system can specifically and successfully meet the challenges posed by growing tumors in cancer patients.
The findings presented in this manuscript implicate critical checkpoints during tumor progression at which therapeutic intervention is necessary, thereby promoting immunotherapeutic control over disease. These include: (i.) optimizing initial enhancement of the priming event against endogenous tumor antigen, (ii.) providing appropriately-timed immunization to prolong the anti-tumor response, and (iii.) monitoring disease at late time points to overcome negative regulation of persisting cells.
Critical Checkpoints During Immunotherapeutic Treatment of Cancer

I. Optimize primary encounter with endogenous tumor antigen
II. Enhance immunization to prolong T cell survival \textit{in vivo}
III. Inhibit negative regulation at late time points

FIGURE 60
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APPENDIX A

*In vivo proliferation assay*

CFSE

TCR-V → TCR-V → SV11

Analyze proliferation in secondary lymphoid tissue

+/− Tag epitope-V only cells

# of cells

CFSE
APPENDIX B

Intracellular Cytokine Assay

splenocytes
ex vivo

+ Tag EPITOPE-V PEPTIDE or CONTROL PEPTIDE

+ Brefeldin A

Incubate 5 hours @ 37°C

Stain for CD8

Fix and permeabilize

Stain for intracellular cytokine (IFNγ)

Analyze by flow cytometry for CD8+IFNγ+ cells
APPENDIX C

In Vivo Cytotoxicity Assay

TCR-V

+/− Tag epitope-V immunization

SV11

7 days

SV11

inject labeled targets i.v.

Flu-NP pulsed CFSE-low

Tag-V pulsed CFSE-high

wait overnight

Analysis

specific loss of labeled targets

CFSE
**APPENDIX D**

**In vitro culture proliferation assay**

TCR-V cells isolated from **Spleen** or **Brain** of SV11 mice 40d post combo-Rx

- Irradiated feeder cells + IL-2
- Varied culture conditions
  - Tag-V peptide
  - PMA/ionomycin
  - Plate-bound αCD3

6 day culture

Analyze by flow cytometry
VITA

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