HOST-CONDITIONING STRATEGIES
FOR ADOPTIVE T CELL IMMUNOTHERAPY OF CANCER

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by
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ABSTRACT

Cancer immunotherapy is poised at the cutting edge of cancer research due to higher response rates and the potential for long-lasting cancer regressions. T cells are critical immune effectors that can identify and eliminate cancerous host cells. Adoptive T cell transfer (ACT) is an emerging immunotherapy in which the patient’s own T cells are isolated and optimized to target the tumor, expanded in culture, and re-infused into the cancer patient. To improve the survival and function of the re-infused T cells, patients are pre-conditioned with chemotherapy or whole-body irradiation (WBI) to deplete host immune cells prior to ACT. Use of host-conditioning regimens significantly improves response and cure rates, but the reasons for failure are unknown. Investigation of the underlying mechanisms of ACT therapy and host-conditioning regimens is necessary to improve the success and applicability of this promising immunotherapeutic approach.

Here, we focus on the influence of WBI and agonist anti-CD40 antibody (which stimulates antigen-presenting cells to activate T cells) host-conditioning regimens on ACT therapy of established murine brain tumors. SV11 mice develop autochthonous tumors of the choroid plexus of the brain due to transgenic expression of the SV40 large Tumor antigen (T Ag) oncoprotein. Using T Ag-specific transgenic T cells, we investigate the underlying mechanisms of WBI and anti-CD40 that promote the in vivo accumulation and persistence of donor T cells during ACT-mediated tumor regression and protection from tumor recurrence. First, we show that WBI conditioning induces a prolonged window of opportunity during which ACT can be administered to mediate regression of established tumors. However, ACT early after WBI is required to achieve maximum survival benefit. Thus, the mechanisms promoting tumor
regression and long-term survival are distinct. We also demonstrate a time-dependent reduction in early donor T cell accumulation when ACT is delayed after WBI that correlates with recovery from host lymphodepletion. Next, we dissect the local and systemic influences of WBI and show that irradiation conditioning can promote successful ACT-mediated tumor regression independently of local irradiation to the brain or tumor. Therefore, the systemic effects of WBI are critical determinants of ACT therapeutic success. Irradiation localized to the body was sufficient to promote long-term survival and donor T cell persistence in the brain. Local irradiation to the tumor site enhanced donor T cell accumulation in the tumor-draining lymph nodes and resulted in modest survival benefit but did not promote tumor regression. Finally, we show that anti-CD40 conditioning can promote ACT-mediated tumor regression in the absence of irradiation, but that only WBI conditioning promotes durable protection from tumor recurrence, demonstrating the differential efficacy of two clinically relevant immunotherapies. Protection from tumor recurrence was associated with the establishment of persistent donor T cells in the brain that were resistant to antibody-mediated depletion. Collectively, these studies illustrate the impact of host-conditioning regimens on ACT therapy and define the requirements relevant to success.
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### LIST OF ABBREVIATIONS

| 459  | transgenic mouse line expressing TCR recognizing T Ag site V |
| ACT  | adoptive T cell transfer                                      |
| ACT-TIL | adoptive T cell transfer with tumor-infiltrating lymphocytes |
| ACT-CAR | adoptive T cell transfer with chimeric antigen receptor T cells |
| Anti-CD8 | depleting monoclonal antibody to CD8                  |
| Anti-CD40 | monoclonal antibody agonist of CD40                      |
| Anti-CD40+ACT | therapeutic regimen of anti-CD40 on days -1 and +1 and ACT on day 0 |
| Anti-CTL-4 | monoclonal antibody antagonist of CTLA-4                  |
| Anti-PD-1 | monoclonal antibody antagonist of PD-1                    |
| Anti-PD-L1/L2 | monoclonal antibody antagonist of PD-L1 or PD-L2          |
| ANOVA | analysis of variance                                       |
| APC  | antigen-presenting cell                                     |
| ATP  | adenosine triphosphate                                      |
| B16  | mouse melanoma cell line                                    |
| B6   | C57BL/6 inbred mice                                         |
| B7   | family of costimulatory molecules                           |
| BI   | body irradiation                                            |
| BSA  | bovine serum albumin                                        |
| β2m  | beta-2-microglobulin, component of MHC class I molecule     |
| CAR  | chimeric antigen receptor                                    |
| CD40L | CD40 ligand                                                 |
| CFSE | carboxyfluorescein succinimidyl ester                       |
| cLN  | superficial cervical lymph nodes                            |
| CNS  | central nervous system                                       |
| CSF  | cerebrospinal fluid                                         |
| CTL  | cytotoxic T lymphocyte                                       |
| CTLA4 | cytotoxic T lymphocyte-associated protein 4                 |
| DNA  | deoxyribonucleic acid                                       |
| DC   | dendritic cell                                               |
| E. coli | *Escherichia coli*                                          |
| F2025 | transgenic mouse line expressing TCR specific for T Ag site IV |
| FACS | fluorescence-activated cell sorting                         |
| FACS Canto II | flow cytometer             |
| FasL | Fas ligand                                                  |
| FBS  | fetal bovine serum                                          |
| Fc   | fragment crystallizable region (tail of antibody)           |
| FDA  | Food and Drug Administration                                |
| FITC | fluorescein isothiocyanate                                  |
| Flt3 | fms-related tyrosine kinase 3                               |
| Foxp3 | forkhead box p3                                             |
| GM-CSF | granulocyte macrophage colony-stimulating factor            |
Gr-1 mouse granulocyte antigen
Gy gray (unit of absorbed radiation)
H-2Db mouse allele for MHC class I molecule
H-2Kb mouse allele for MHC class I molecule
HEPES 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (a buffer)
HLA human leukocyte antigen
H&E hematoxylin and eosin
HI head irradiation
HMGB-1 high-mobility group box-1
HPE homeostatic peripheral expansion
ICD immunogenic cell death
IDO indoleamine-2,3-dioxygenase
IFN interferon
IgG immunoglobulin G
IL interleukin
IL-2Ra IL-2 receptor chain α
IL-12R IL-12 receptor
IL-15Rα IL-15 receptor chain α
IL-15Rβ IL-15 receptor chain β
i.p. intraperitoneal
IPV inactivated polio vaccine
i.v. intravenous
KLRG1 killer cell lectin-like receptor G1
LAG3 lymphocyte activation protein 3
LSRFortessa flow cytometer
LSRIISORP flow cytometer
MAGE-1 melanoma family antigen A1, member of MAGEA gene family
MART-1 melanoma antigen recognized by T cells 1
MDSC myeloid-derived suppressor cell
MFI mean fluorescence intensity
MHC major histocompatibility complex
MPEC memory-precursor effector cell
mRNA messenger ribonucleic acid
NBF neutral buffered formalin
NCT national clinical trial
NK natural killer cell
NY-ESO-1 New York Esophageal Squamous Cell (cancer-testis antigen)
p53 (TP53) tumor protein (EC :2.7.1.37) (tumor suppressor)
PAP prostatic acid phosphatase
pAPC professional antigen-presenting cell
PBS phosphate-buffered saline
PD-1 programmed cell death protein 1
PD-L1/L2 programmed cell death protein ligand 1 / ligand 2
poly:ICLC polyinosinic-polycytidylic acid, carboxymethylcellulose, poly-L-lysine (double stranded RNA, TLR3 ligand)
pp2A protein phosphatase 2
pRb phosphorylated retinoblastoma (tumor suppressor)
pSV11 plasmid used to derive SV11 mouse line
RBC  red blood cell
RIP-Tag  rat insulin promoter-T antigen (pancreatic cancer model)
RPMI  Roswell Park Memorial Institute
SCID  severe combined immunodeficiency
SLEC  short-lived effector cell
STING  stimulator of interferon genes
SV11  transgenic mouse line on the C57Bl/6 genetic background that develop autochthonous choroid plexus tumors due to the transgenic expression of full-length T Ag from the SV40 viral promoter
SV40  simian virus 40
T Ag  SV40 large T antigen
TCR  T cell receptor
TCR-gB  transgenic TCR that recognizes herpes simplex glycoprotein B
TCR-I  Line 416 mice expressing a transgenic T cells recognizing T Ag site I
TCR-IV  Line F2025 mice expressing a transgenic TCR that recognizes T Ag site IV
TCR-V  Line 459 mice expressing a transgenic TCR that recognizes T Ag site V
Tetramer-gB  gB/Kb MHC tetramer
Tetramer-IV  IV/Kb MHC tetramer
TGFβ  transforming growth factor beta
TIL  tumor-infiltrating lymphocyte
TLR  toll-like receptor
TME  tumor microenvironment
TNFα  tumor necrosis factor α
TREG  regulatory T cell
TRM  resident-memory T cell
VCAM-1  vascular cell adhesion molecule-1
VV  vaccinia virus
VV-941T  recombinant vaccinia virus expressing full-length T Ag
WBI  whole-body irradiation
WBI(day -1)+ACT  therapeutic regimen of WBI on day -1 followed by ACT on day 0
WT-19  immortalized cell line expressing full-length T Ag (syngeneic with B6 mice)
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Those who have not been trained in chemistry or medicine, which after all is only applied chemistry, may not realize how difficult the problem of cancer treatment really is. It is almost – not quite, but almost – as hard as finding some agent that will dissolve away the left ear, say, and leave the right ear unharmed. So slight is the difference between the cancer cell and its normal ancestor.

-William Woglom, American physician and cancer researcher
Chapter 1

Introduction

Historical links between immunity and cancer

For millennia, the search for the causes and cures of cancer has intrigued and confounded physicians. The oldest known medical text – the *Edwin Smith Papyrus* (circa 3000 BC) – contains a description of a patient with a breast tumor (1). Another ancient medical text ascribed to the Egyptian physician Imhotep – the *Papyrus Ebers* – offers recommendations on the treatment of cancer, among other ailments. Specifically, it is written that an incision into the tumor will allow infection of the tumor, leading to its regression (2). This treatment, however primitive, suggests ancient physicians were well aware of the phenomenon of immune-mediated tumor regression.

For most of human history, the diagnosis of cancer has held a grim prognosis, although spontaneous tumor regressions are occasionally, albeit rarely, observed (3,4). The protection afforded by febrile infection against cancer has been appreciated for hundreds of years. In 1725, French physician Deidier observed a reduction in cancer incidence in prostitutes infected with syphilis (reviewed in (5)). In his 1855 essay titled “The Diagnosis of Surgical Cancer”, British surgeon John Z. Laurence wrote “As a rule it will be found that cancerous patients have been otherwise remarkably free from disease”, suggesting that patients with a history of infections may actually be protected from cancer.

In the 19th century, multiple physicians attempted to exploit the relationship between fever and tumor regression for therapeutic purposes (reviewed in (5)). Most notable was American surgeon William B. Coley, who in 1891 began injecting his patients’ tumors with
mixtures of bacterial products (primarily *Streptococcus erysipelatos* and *Bacillus prodigiosus*, now called *Streptococcus pyogenes* and *Serratia marcescens*, respectively) with the intent of inducing fever and subsequent tumor regressions. Indeed, Coley observed tumor regressions in a fraction of his patients in association with fever induction (6). Coley has been called the “Father of Immunotherapy” because of his innovation and dedication to the development of this approach. In total, Coley treated close to 1000 cancer patients by injection of bacterial products, which came to be known as Coley’s toxins and were commercially available to physicians at one time (7). Despite his successes, the use of Coley’s toxins did not gain widespread acceptance in the medical community, and this approach was quickly overshadowed by newly developed radiation therapy and chemotherapy. Nevertheless, the early investigation of cancer immunotherapies by Coley and others provided evidence that the approach could be successful and set the stage for future generations of immunotherapy research.

**References**


Chapter 2

Review of the literature

Tumor immunology

The concept that the immune system can identify and eliminate cancerous cells was formalized over 50 years ago as the cancer immunosurveillance hypothesis. Decades of research have yielded evidence in support of this hypothesis, as well as the appreciation that a growing tumor represents an ongoing evasion of immunosurveillance.

The cancer immunosurveillance hypothesis

Clinical evidence to support the cancer immunosurveillance hypothesis

The idea that tumor cells can be distinguished from healthy host cells and eliminated by the immune system has been postulated for many years, however, the general acceptance of tumor immunosurveillance as a responsibility of the host immune system was only gained relatively recently. In 1909, Paul Ehrlich suggested that the immune system may play a role in protecting hosts from tumor development (1). Five decades later, Burnet and Thomas collectively formulated a hypothesis of cancer immunosurveillance in which tumor cells acquired antigenic properties detectable by the immune system – a primary host defense mechanism against neoplasia (2,3). In 1957, Burnet wrote: “It is by no means inconceivable that small accumulations of tumor cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction, with regression of the tumor and no clinical hint of
its existence”. This hypothesis was later supported by data from an analysis showing high rates of cancer incidence in genetically immune-suppressed patients (4). Similar findings were observed in immune-suppressed patients following organ transplantation (5).

In 1991, identification of the first human tumor antigen was a landmark finding in tumor immunology (6). This antigen was identified from a melanoma patient as a non-mutated gene product of the MAGEA-1 gene, which is typically silent on normal tissues but is expressed in a fraction of melanomas and other tumors. Importantly, this antigen was shown to be expressed by melanoma samples from multiple patients, demonstrating a shared tumor antigen that could be targeted therapeutically. Many more tumor antigens have now been identified (7). Tumors antigens can be classified into four groups by source: (i) mutated gene products; (ii) oncoviral proteins; (iii) expression of germline genes typically restricted to germline cells that do not express MHC molecules (often called cancer-germline or cancer-testis antigens); and (iv) overexpressed non-mutated self-proteins (also called differentiation antigens) (8). The identification of tumor antigens in human tumors (as well as T cells that recognized the tumor antigens) provided solid evidence to support the immunosurveillance hypothesis in humans.

**Experimental evidence to support the cancer immunosurveillance hypothesis**

In 1957, it was demonstrated that mice immunized with carcinogen-induced tumor cells could reject subsequent tumor inoculations (9), suggesting tumor cells express specific antigens that can be detected by the immune system. A 1974 study by Osias Stutman experimentally tested the cancer immunosurveillance hypothesis by comparing the rates of carcinogen-induced tumorigenesis in immune-deficient athymic-nude mice, which lack mature T cells, versus their immune-competent counterparts (10). Surprisingly, Stutman found no evidence of enhanced tumorigenesis in immune-deficient mice. While this result did not disprove the cancer
immunosurveillance hypothesis, it did cast considerable doubt, as well as suggest that tumor antigenicity was a property of experimentally carcinogen-induced, but not spontaneously-arising tumors. This result by Stutman was later contradicted by studies from other groups described below. This discrepancy was explained by the finding that a small population of mature T cells can be found in athymic nude mice and would be expected to provide immunosurveillance.

A breakthrough in 1982 showed that spontaneous tumors did in fact possess tumor antigens (11). The investigators cultured a non-immunogenic spontaneously-arising murine leukemia cell line in a mutagenizing agent. The unknown mutations acquired by the leukemia cells rendered the mutant cells immunogenic. Mice immunized with the mutant leukemia cells were protected from subsequent inoculation with the parental cell line, demonstrating that the parent leukemia cells possessed tumor antigens that were shared with the mutant leukemia cells and proving that spontaneously-arising tumors possessed tumor antigens that could be targeted by the immune system.

In 1994, a study demonstrating the importance of host-derived IFNγ in immune-mediated tumor rejection showed that tumors rendered insensitive to IFNγ signaling could evade immunosurveillance. The authors showed that an immunogenic tumor cell line, which could be rejected by immune-competent mice, was rendered non-immunogenic when it was engineered to express a dominant-negative IFNγ receptor. Thus, the parental tumors were rejected by a mechanism involving host-derived IFNγ and the IFNγ receptor on the tumor cells. The IFNγ-insensitive tumors grew faster in naïve mice and in mice immunized with the parent tumor cell line compared to IFNγ-sensitive tumors (12). A follow-up study by the same research group showed that both carcinogen-induced and spontaneously-arising tumors grew with greater frequency in mice insensitive to IFNγ due to genetic knockout of the IFNγ receptor (13). A similar role for the pore-forming protein perforin was shown in immunosurveillance (14).
A seminal work in 2001 showed that progressing tumors evolve to resist rejection by the host immune system. In this study, tumors grown in immune-competent mice could be passaged to secondary recipient immune-competent mice and continue to grow. Conversely, when tumors grown in immune-deficient mice were passaged to secondary recipient immune-competent mice, a significant fraction of these tumors were rejected by the competent immune system (15). This process of tumor evolution to evade the host immune system was termed immunoediting and will be described in detail below.

**Tumor resistance mechanisms to immunosurveillance**

As discussed above, the cancer immunosurveillance hypothesis posits that the immune system protects the host by eliminating cancerous or pre-cancerous cells through recognition of aberrant changes in surface molecule expression, such as T cell recognition of neoantigens on genetically unstable cancerous cells (Fig. 2-1). Failure of immunosurveillance to eliminate dangerous cancerous or pre-cancerous cells, therefore, may lead to the development of tumors that become progressively more resistant to endogenous anti-tumor immune responses. A clinically apparent tumor has likely utilized a number of evasion mechanisms to escape immunosurveillance.

**Tumor cell intrinsic resistance to immunosurveillance**

T cells identify tumor targets by recognition of tumor antigens presented by MHC class I molecules, thus tumor cells that minimize or abandon expression of these surface molecules render themselves invisible to T cells. Antigen-specific evasion of T cell-mediated immunity has been shown following loss of target antigen expression by tumor cells in animal models and
human patients (16,17). Expression of HLA or β2-micro-globulin (β2m) is also commonly disrupted in tumor cells due to accumulating mutations or loss of heterozygosity (18), and the frequency of disruption correlates with tumor progression and resistance to immunotherapy (19,20). Natural killer (NK) cells are innate lymphoid cells that can eliminate tumor cells that lose MHC class I expression and therefore represent an important component of immunosurveillance (21). NK-mediated cytotoxicity is controlled by a balance of activating and inhibitory receptors, the latter of which is engaged by MHC molecules on healthy cells but not on MHC-negative tumor cells. Interestingly, NK cells can lose expression of activating receptors in the presence of tumor cells (22), resulting in tumor cell evasion of NK-mediated cytotoxicity. The exact mechanisms mediating this evasion remain incompletely defined.

Cytotoxic T lymphocytes (CTLs) employ multiple effector mechanisms that induce tumor cell apoptosis. To evade immunosurveillance, tumor cells become resistant to immune-mediated apoptosis. CTLs produce the effector cytokine IFNγ, which induces tumor cells to express the Fas death receptor. Subsequent engagement of Fas by Fas-ligand-expressing effector cells induces tumor cell-apoptosis (23). Tumor cells disrupt Fas signaling, for instance, by reducing receptor expression (24) or expressing mutant Fas receptors (25). CTLs also produce apoptosis-inducing granzyme B, which gains access to target cells in conjunction with pore-forming perforin. Tumor cells have been shown to increase expression of a protease inhibitor that inactivates granzyme B; interestingly, this mechanism is normally utilized to protect T cells from self-granzyme B (26). In addition to neutralizing immune-mediated apoptotic mechanisms, tumor cells alter the intracellular balance of pro- and anti-apoptotic proteins to resist apoptosis and favor their own survival (27). Thus, tumor cells are able to evade immune recognition and immune-mediated elimination via a number of tumor cell intrinsic mechanisms.
**The immunosuppressive tumor microenvironment**

Tumors are composed of not only cancer cells, but also associated stromal cells including myeloid cells and lymphocytes recruited to the tumor site. Collectively, the stroma supports the growing tumor. In particular, the stroma suppresses immunosurveillance by establishing an immunosuppressive tumor microenvironment (TME) (Fig. 2-2) (28).

Tumors modulate their surroundings by producing a diverse mixture of chemokines, cytokines, and enzymes, resulting in the recruitment and differentiation of immune cells including myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T\textsubscript{REG}), which establish and maintain an immunosuppressive TME (21,29). Local production of cytokines such as TGF-β can directly inhibit effector T cell function and promote the function of T\textsubscript{REG} cells. In addition, the TME is rich in immunosuppressive enzymes such as indoleamine 2,3-dioxygenase (IDO) and arginase I, which catalyze the degradation of tryptophan and arginine, respectively, reducing the availability of metabolites critical for T cell activity (30,31). In mouse tumor models, the accumulation of MDSCs, a heterogeneous population of immature myeloid cells, has been shown to contribute to the immunosuppressive TME. MDSCs express arginase I and inducible nitric oxide synthase, resulting in inhibition of T cell proliferation and effector function, respectively (32). The production of nitric oxide in the TME has also been shown to chemically modify and inactivate chemokines, resulting in decreased T cell recruitment to the tumor (33). The role of MDSCs in human cancer progression remains less established. An increase in MDSC levels has been observed in the peripheral blood of patients with pancreatic, esophageal, and gastric cancers (34). However, a study in melanoma patients found no increase in the levels of circulating MDSC and showed that tumor-derived myeloid cells displayed impaired immunosuppression compared with circulating myeloid cells (35). Thus, the influence of MDSC-mediated immunosuppression may differ by tumor type and tissues analyzed.
CD4^+CD25^+FoxP3^+ T_{REG} cells are essential for the maintenance of tolerance toward self-antigens and are frequently detected within tumors. The increased frequency of T_{REG} cells in tumors has been associated with a poor prognosis (36). These cells have been shown experimentally to inhibit effector T cell responses using a variety of mechanisms, including both contact-dependent and independent processes (37). For example, constitutive expression of the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) inhibitory receptor, which binds B7 costimulatory ligands with high affinity, enables T_{REG} cells to interfere with T cell access to costimulation (38). T_{REG} cells also consume available IL-2 by expressing the high-affinity receptor IL-2Rα, depriving effector T cells of this important cytokine (39). Additionally, T_{REG} cells secrete suppressive cytokines, such as TGF-β, which directly inhibit effector T cell function (40). The immunosuppressive role of T_{REG} cells in human cancer is highlighted by the reproducible finding that a high ratio of CD8^+:T_{REG} cells correlates with patient survival in multiple cancers (41,42).

Tumor-infiltrating effector T cells commonly express inhibitory receptors (43), rendering them susceptible to inhibition in the TME. In particular, ligation of the programmed death-1 inhibitory (PD-1) receptor, expressed by activated T cells (44), results in negative regulation of effector functions including proliferation and cytokine production (45). Two PD-1 ligands have been identified, PD-L1(46) and PD-L2 (47), and expression of these ligands has been observed in both lymphoid and non-lymphoid tissues (48), including expression of PD-L1 in human tumors (49). PD-L1 expression in tumors is not constitutive but instead is induced in response to IFNγ and other cytokines produced by tumor-infiltrating effector cells (50,51). Thus, the expression of PD-1 ligands by tumor cells represents a reaction to suppress anti-tumor immunity. Additional T cell inhibitory receptors have been identified (52) and shown to mediate suppression of tumor-reactive T cells (53-55).
In summary, the cancer immunosurveillance hypothesis suggested that the immune system protects against the development of tumors by identifying and eliminating cancerous or pre-cancerous cells. However, a subset of cancerous cells evade immunosurveillance and develop into a tumor with an immunosuppressive TME.

The cancer immunoediting hypothesis

In 2001, the research group of Robert Schreiber published a study that provided definitive evidence that progressing tumors continuously evolve to evade the immune system (15). In this study, groups of immune-competent and immune-deficient mice were administered a carcinogen to induce tumor development. Tumors that grew out were removed and subsequently injected into groups of immune-competent secondary recipient mice. All tumors derived from the immune-competent “donor” mice grew progressively in immune-competent secondary recipients (Fig. 2-3a). Interestingly, 60% of tumors derived from the immune-deficient donor mice were rejected by immune-competent secondary recipients (Fig. 2-3b); 40% of the tumors derived from immune-deficient donor mice successfully grew in immune-competent secondary recipients (Fig. 2-3c). Thus, the breakdown in immunosurveillance required for tumor growth does not necessarily constitute a loss of anti-tumor immunity. Instead, Schreiber et al. hypothesized, the ongoing anti-tumor immune response imposes an evolutionary pressure that results in a less immunogenic and, paradoxically, more aggressive tumor. This process was termed “cancer immunoediting”.

The cancer immunoediting hypothesis was later formalized into three phases termed Elimination, Equilibrium, and Escape (56). The Elimination phase begins when immune cells detect the emergence of cancerous or pre-cancerous cells and initiate a local inflammatory response; it ends when the dangerous cells are eliminated. Occasionally, cancerous cells evade immunosurveillance and begin to form a tumor. The growth of the developing tumor may become
arrested by an immune response. This stable balance between tumor growth and active anti-tumor immunity is termed the Equilibrium stage, and may carry on for extended periods of time—perhaps even the lifespan of the host. There is experimental evidence to support the notion of an equilibrium between tumors and immunity. A follow-up study from Schreiber’s group showed that when mice were treated with a low dose of carcinogen, they did not develop visible tumors. However, when the T cells of these mice were later depleted (by antibody), detectable tumors grew at the site of carcinogen application (57). Presumably, occult cancer cells had been held in equilibrium by the host immune system, and this equilibrium was lost upon T cell depletion. A tumor that has eluded immune equilibrium has reached the Escape phase. A tumor in the Escape phase has been “edited” to evade anti-tumor immunity utilizing a number of evasion mechanisms as described earlier including loss of antigenicity or increased resistance to apoptosis. A tumor may also reach the Escape phase if equilibrium is lost due to immune deficiency caused by aging, illness, or immunosuppressant therapy. Tumors that have become clinically detectable have most likely reached the Escape phase.

The immune system employs numerous mechanisms to protect the host from cancer. The immune system detects and engages neoplasia, from pre-cancerous cells to invasive tumors. In response, the cancer evolves and evades. A growing understanding of this interplay has guided the conception, development, and success of modern cancer immunotherapies.
Cancer immunotherapy

Overview

The goal of cancer immunotherapy is to promote anti-tumor immune responses (58), and a number of different immunotherapies are under investigation to achieve this goal. Some immunotherapies target the tumor directly by generating tumor-specific immunity, while other immunotherapies non-specifically promote anti-tumor immunity using stimulatory cytokines or antibodies to modulate immune signaling pathways.

A primary advantage of immunotherapies over standard cancer therapies such as surgery, chemotherapy, and radiation therapy is the high specificity with which immune cells and molecules can distinguish and identify targets. The term “immunotherapy” is applied to a multitude of anti-cancer agents. These agents can be broadly classified as antibody therapies, cytokine therapies, vaccines, and cell therapies. Monoclonal antibody therapies take advantage of the high specificity and affinity of antibodies to bind target antigens. These targets include molecules on the surface of tumor cells or immune cells that can be targeted for elimination of a specific cell population. For example, the monoclonal antibody rituximab (anti-CD20) is used for the treatment of B cell malignancies because it binds CD20 on the surface of B cells, resulting in tumor cell elimination by multiple mechanisms, including direct induction of B cell apoptosis (59). Additionally, receptors and ligands that promote tumor growth can be targeted, such as the vascular endothelial growth factor-A, which is targeted and blocked by the monoclonal antibody bevacizumab, depriving tumor cells of this growth factor (60). Monoclonal antibody therapies are also used to modulate immune cell signaling by blocking or ligating cell surface receptors on immune cells and tumor cells. Antibodies that block immune-regulatory signaling pathways, called “checkpoint inhibitors”, have garnered particular interest recently (61). Selected
monoclonal antibody therapies will be discussed in the section titled “Immune-modulating antibodies”. Cytokine therapies, such as the T cell growth factor IL-2, also promote anti-tumor T cell responses. Selected cytokine therapies will be discussed in the section title “Immune-modulating cytokines”. Both cytokines and monoclonal antibodies that aim to promote anti-tumor T cell responses are relatively non-specific, as they modulate T cell activity in the host without reverence to tumor-specificity. Therapeutic cancer vaccines promote tumor-specific immunity through administration of tumor antigens in a variety of forms, including peptides and tumor cell lysates. These vaccines will be discussed in the section titled “Cancer vaccines”. Cell therapies utilize autologous cells that are manipulated ex vivo to target tumor antigens and re-introduced to the patient to induce anti-tumor T cell responses. In particular, cell therapies that utilize autologous T cells will be discussed in detail in the section titled “Adoptive T cell transfer therapy”.

In general, immunotherapies that promote anti-tumor T cell responses are particularly promising because they can induce an ongoing immune response that regresses established tumors and protects from future recurrence (62). Some T cells are capable of distinguishing tumor cells from healthy cells with high specificity and can directly eliminate tumor cells and recruit additional immune effector cells to the tumor site. Following elimination of the tumor, T cells can form memory populations that persist for the life of the host and protect against future tumor recurrence. Thus, T cells represent an ideal agent that can survey the host and identify and eliminate tumor cells, including metastases. This section will describe a variety of immunotherapies that promote anti-tumor T cell responses. Those immunotherapies that aim to induce T cell immunity against the tumor by infusing tumor-reactive lymphocytes (adoptive T cell transfer) or vaccinating with tumor antigens to generate endogenous anti-tumor immunity (cancer vaccines) may represent the most potent immunotherapies, as they promote anti-tumor T cell responses that target the tumor directly in an antigen-specific manner.
Adoptive T cell transfer therapy

Adoptive T cell transfer (ACT) therapies utilize autologous T cells that are isolated ex vivo and refined in the laboratory prior to re-infusion into the cancer patient. Currently, multiple ACT protocols are under investigation with the common goal of promoting T cell-mediated tumor regression by enhancing the number and function of tumor-reactive T cells in the cancer patient.

The use of tumor-infiltrating lymphocytes for ACT therapy

In 1921, the correlation between the presence of tumor-infiltrating lymphocytes (TIL) and prolonged survival was observed in gastric carcinoma patients. (63,64). In decades that followed, a putative role for lymphocytes (and other hematological cells) in “host resistance” against tumors was suggested (65,66), and the correlation between lymphocytic infiltration and positive prognosis became well-established in a variety of tumors (67-71). Thus, the potential utility of lymphocytes in the therapy of cancer was recognized. In 1964, it was shown that established tumors were treatable by adoptive transfer of lymphocytes from tumor-immunized rats into tumor-bearing rats (72). These findings, however, could not be directly translated to therapies for cancer patients without a human source of tumor-reactive T cells. As laboratory cell culture techniques were further refined, TIL emerged as a promising source of autologous tumor-reactive T cells.

Soon after the first techniques were developed to maintain mouse (73) and human (74) lymphocytes in culture, the identification of a soluble T cell growth factor, produced by activated lymphocytes (75) and later named IL-2, proved crucial for the advancement of adoptive T cell transfer therapy. A study by Gillis and Smith showed that murine T cells could be expanded in
culture using T cell growth factor, and that these cells maintained their antigen-specificity despite extended time in culture (76). Similar findings were later shown using human T cells isolated from peripheral blood (77). Attempts to isolate and grow significant numbers of TIL had been largely unsuccessful until 1980, when experiments in mice showed that TIL populations preferentially expanded when excised tumor tissue was cultured with the recently discovered T cell growth factor (78), demonstrating the ability to generate large numbers of tumor-reactive autologous T cells. Very soon thereafter, it was shown that autologous lymphocytes could be safely re-infused into human patients following \textit{ex vivo} expansion in T cell growth factor (79). These findings unveiled the potential to isolate, expand, and re-infuse TIL to cancer patients.

Early ACT therapy clinical trials were designed based on pre-clinical data from mouse tumor models that showed TIL could be isolated from established tumors, expanded in culture, and re-introduced into tumor-bearing mice to mediate tumor regression (80). To isolate TIL, a fresh tumor specimen was dissected into small (< 5mm$^3$) pieces and enzymatically digested to achieve a cell suspension, which was then cultured in the presence of IL-2 to favor expansion of lymphocytes over tumor cells. The expanded TIL product was predominantly composed of Lyt-2$^+$ (CD8$^+$) T cells, and was shown to more potently and specifically mediate tumor cell lysis \textit{in vitro} compared to bulk lymphocytes activated with IL-2. Maximal success of this therapeutic protocol required pre-treatment of the recipient host with lymphodepleting chemotherapy or whole-body irradiation (WBI) before ACT, as well as the systemic administration of IL-2 following ACT. Soon after ACT therapy using TIL (ACT-TIL) was shown to successfully mediate tumor regression in mice, a similar protocol for TIL isolation and expansion from human tumors was established (81), and the stage was set for ACT-TIL trials in human cancer patients.

Initial ACT-TIL clinical trials were performed under the direction of Steven A. Rosenberg at the National Cancer Institute. An initial pilot study in patients with various cancers demonstrated the safety of ACT-TIL therapy, as well as proper dosages of cyclophosphamide,
TIL, and systemic IL-2 (82). The ACT-TIL protocol was established as follows: patients first underwent surgery to remove tumor tissue required for TIL culture and expansion; patients then received host-conditioning with lymphodepleting chemotherapy; host-conditioning was followed by TIL infusion one to two days later; immediately following TIL infusion, patients began receiving systemic IL-2 therapy (Fig. 2-4). Twenty metastatic melanoma patients were initially treated using this ACT-TIL protocol. Objective regressions were observed in 11/20 patients (83). The investigators continued to recruit additional patients, and by 1994, ACT-TIL therapy had been administered to 86 metastatic melanoma patients. Overall, the objective response rate of ACT-TIL therapy in metastatic melanoma patients was 34% (84), a dramatic improvement over other therapies available to metastatic melanoma patients at the time (85).

Following demonstration that ACT-TIL therapy was safe and highly effective compared to alternative therapies, additional pre-clinical and clinical studies were required to overcome significant limitations to the success and applicability of ACT therapy. In particular, approximately two-thirds of treated patients still saw no objective regression of their cancer, and many of the observed regressions were transient. New approaches to generate tumor-reactive T cells for ACT therapy would be needed to apply this therapy to patients for whom a TIL product could not be generated. Finally, the applicability of this protocol to cancers other than melanoma remained unknown. Therefore, additional clinical and pre-clinical studies were performed to improve the frequency and duration of tumor regressions, as well as applicability to other cancers and patients without available TIL.

**The importance of lymphodepleting host-conditioning regimens for ACT therapy**

Perhaps the most critical advancement in ACT-TIL therapy since the first clinical trials has been the use of lymphodepleting host-conditioning regimens with increasing intensity. The
use of lymphodepleting host-conditioning regimens to improve ACT therapy had been demonstrated previously in animal models (80,86-88). Host-conditioning in early ACT-TIL trials consisted of a single dose of cyclophosphamide prior to ACT and did not demonstrably impact therapeutic outcomes (84). A landmark study in 2002 demonstrated that a more intense regimen of lymphodepleting chemotherapy promoted the in vivo expansion and persistence of tumor-specific T cells, in association with an objective response rate over 50%, following ACT-TIL therapy in metastatic melanoma patients (89). In follow-up studies, chemotherapy was combined with whole-body irradiation (WBI) to induce even greater lymphodepletion prior to ACT-TIL therapy (90,91). The combination of chemotherapy with 12 Gy WBI (the highest dose tested) resulted in a staggering 72% objective response rate to ACT-TIL therapy in metastatic melanoma patients, including a 40% complete response rate (91). Furthermore, responses were durable, with 9/10 complete responses ongoing at three years and beyond. Other institutions have replicated the use of ACT-TIL therapy in metastatic melanoma patients with similar success (92-94).

Collectively, these trials in human patients have established both the promise of ACT-TIL as a curative therapy and the crucial role that lymphodepleting host-conditioning regimens play in achieving optimal outcomes.

Studies in experimental mouse models have elucidated a number of mechanisms by which lymphodepleting host-conditioning regimens may contribute to improved outcomes using ACT therapy (Fig. 2-5 to 2-7). These mechanisms can be considered to fall into two broad categories: first, the elimination of endogenous host cells that may directly or indirectly suppress the anti-tumor effect of adoptively-transferred T cells; and second, the activation of host innate immune cells that support the anti-tumor effect of adoptively-transferred T cells. As discussed previously, a number of immunosuppressive, tumor-promoting cell populations have been described in tumor-bearing hosts (Fig. 2-2). The use of increasingly intense regimens of WBI conditioning to tumor-bearing mice prior to ACT therapy resulted in profound lymphodepletion
of host immune cells (Fig. 2-5), including potentially suppressive CD4$^+$ T cells (including $T_{\text{REG}}$ cells) and Gr-1$^+$ myeloid cells (including MDSCs), and correlated with improved therapeutic efficacy (95). Lymphodepletion can also increase access to important homeostatic cytokines, such as IL-7 and IL-15, through the removal of endogenous cytokine sinks (Fig. 2-6), namely T and NK cells, resulting in increased efficacy of ACT therapy (96). Host-conditioning regimens have additional effects on the tumor-bearing host beyond lymphodepletion, which may also support anti-tumor immunity. The direct cytotoxic effects of chemotherapy and ionizing radiation can result in the death of tumor cells, initiating a process termed immunogenic cell death, in which tumor antigens are released by the dying tumor cells along with cellular components that act as “danger signals” and stimulate host APCs to present tumor antigens to anti-tumor T cells (Fig. 2-7) (97-99). The translocation of gut-derived microbial products into systemic circulation via irradiation-induced micro-perforations provides an additional source of APC-stimulating danger signals that support anti-tumor immunity (Fig. 2-7) (100). The direct effects of irradiation can induce host DCs to support anti-tumor immunity through up-regulation of costimulatory molecules and expression of IL-12 (101,102). Collectively, these studies illustrate the multitude of effects the host-conditioning regimens used for ACT therapy have on the host immune system.

**Genetically engineered T cells for ACT therapy**

An important limitation of ACT-TIL therapy is the requirement for autologous, pre-existing tumor-reactive T cells that can be isolated and expanded in culture. Thus, additional sources of autologous T cells may serve to overcome this limitation and provide useful T cells for ACT therapy.
**TCR-transgenic T cells**

One method to generate tumor-reactive T cells involves the use of retroviral vectors encoding TCR αβ chains specific for known tumor antigens into autologous T cells, thus allowing a non-reactive T cell to be redirected to recognize a tumor antigen. Polyclonal human peripheral blood lymphocytes have been shown to express a transgenic TCR following retroviral transduction with TCR αβ chains cloned from a highly reactive, melanoma-specific T cell (103). The transduced cells secreted IFNγ and GM-CSF in response to specific antigen recognition. Importantly, this technique was also successful in redirecting autologous TIL to recognize tumor antigen, demonstrating the applicability of retroviral TCR-transduction to redirect previously unreactive TIL. Additional tumor antigens have been successfully targeted using redirected TCR-transduced lymphocytes, including the common tumor antigens NY-ESO-1 and p53 expressed by many tumor types beyond melanoma (104,105). In clinical trials, ACT of autologous T cells redirected to recognize the MART-1 and NY-ESO-1 tumor antigens has been shown to mediate objective responses in patients with melanoma and synovial cell sarcoma, including complete responses in association with persistence of the transduced T cells at 1+ year post-ACT (106-108). Objective response rates targeting MART-1 and NY-ESO-1 were 30% and ~50%, respectively. It is important to note that targeting the MART-1 antigen resulted in autoimmune toxicities due to expression of MART-1 in healthy tissues such as eye, ear, and skin (107). Meanwhile, targeting NY-ESO-1 resulted in no off-target toxicity, as the only healthy tissue known to express NY-ESO-1 is cells of the testes, which do not express MHC molecules and therefore are not targeted (108). These trials demonstrate promise for the future of TCR-redirected T cells in ACT therapy. In particular, use of TCR-redirected T cells may benefit patients for whom TIL cannot be isolated or expanded.
**Chimeric antigen receptor T cells**

A second source of autologous tumor-reactive T cells can be obtained through the transgenic expression of chimeric antigen receptors (CARs). CARs are cell surface fusion molecules comprised of an extracellular antigen-recognizing domain fused to an intracellular signaling domain that triggers T cell effector functions upon antigen engagement. The extracellular antigen-recognizing domain is a single chain variable fragment derived from a monoclonal antibody with high affinity for the target antigen. Thus, CARs recognize target antigen in an MHC-independent manner. The intracellular signaling domain was originally comprised of the intracellular portion of the CD3-ζ chain that triggers T cell activation signaling. More recently, additional signaling motifs have been added to the intracellular domain to improve cytokine production, cell proliferation, and anti-tumor efficacy (109,110). Similar to redirected TCR-transgenic T cells, CAR T cells are generated through transduction of a CAR-encoding viral vector into autologous peripheral blood-derived lymphocytes. In a severe-combined immunodeficiency (SCID) mouse model of metastatic prostate cancer, CAR-transduced human T cells were shown to successfully treat established cancer using an ACT therapeutic approach (ACT-CAR) that included host-conditioning and IL-2 (111). In clinical trials, ACT-CAR therapy targeting the CD19 B cell antigen has been used to successfully treat B cell malignancies in adult and pediatric patients (112,113). Clinical trials of ACT-CAR therapy for solid tumors in human patients have not yet been published, but trials are ongoing and success has been reported in animal models (114).
Future challenges for ACT therapy

A number of challenges remain for the treatment of cancer with ACT therapy (115). To date, this therapy has been primarily evaluated in metastatic melanoma patients. Successful ACT therapy in autochthonous tumor models of prostate cancer and brain cancer has been demonstrated (116,117). Whether ACT therapy can be successfully applied to a wide variety of cancers in humans remains to be determined, although efficacy in synovial sarcoma and B cell malignancies has been demonstrated using genetically engineered T cells (108,112,113). A significant impediment to wide-ranging application of ACT-TIL therapy is the dependence on *ex vivo* isolation of tumor-reactive TIL from tumor specimens. This impediment can be overcome using TCR-redirected T cells or CAR T cells, however these techniques are currently limited to targeting known tumor antigens. One benefit of ACT-TIL therapy is the personalized nature of the expanded TIL product that allows for the targeting of many unique neoantigens simultaneously. Thus, the development of more sensitive techniques to isolate and expand reactive TIL from less immunogenic tumors may result in wider application of ACT-TIL therapy (118).

A second challenge that warrants investigation is the identification of reliable biomarkers to predict (for example): the likelihood of a patient response (or non-response) to ACT therapy; the likelihood of *in vivo* TIL efficacy (versus *in vitro* reactivity, which does not necessarily correlate with response); and the likelihood of tumor relapse. Reliable biomarkers will allow for the development of more potent TIL products and better personalization of the ACT protocol to individual patient needs. Currently, a patient must be able to physically withstand host-conditioning regimens and IL-2 therapy in order to receive ACT therapy. These requirements exclude a large fraction of patients from eligibility; for example, those with heart disease may be unable to withstand the cardiovascular stress induced by IL-2 therapy. Reliable biomarkers will
allow for the selection of appropriate host-conditioning regimens and cytokine support that are necessary for therapeutic success but can also be minimized if deemed unnecessary by biomarker predictions. Currently, the best response rates to ACT therapy are achieved using an intense conditioning regimen of chemotherapy and 12 Gy WBI (which requires concurrent bone marrow transplant) (91). Whether such an intense regimen is required for all patients is unknown. In the current study, we investigate the underlying mechanisms of host conditioning regimens that promote successful ACT therapy. A more thorough understanding of the mechanisms of host-conditioning will allow for improved responses to ACT therapy and a more targeted approach to host-conditioning that limits the off-target effects of chemotherapy and irradiation, potentially forgoing the use of these agents altogether.

**Cancer vaccines**

Cancer vaccines aim to generate immune responses against tumor-specific antigens through administration of antigen in various forms: whole tumor cells; recombinant or synthetic tumor antigen protein or peptides; antigen-loaded autologous APCs; and viral vectors expressing known tumor antigens. Hundreds of cancer vaccine clinical trials have been conducted in a variety of solid cancers, often resulting in the induction of detectable anti-tumor immunity (119). Unfortunately, detectable anti-tumor immunity does not necessarily translate to tumor regression and clinical benefit (120). A recent meta-analysis reviewed a total of 936 cancer patients treated using various cancer vaccinations and observed an overall objective response rate of only 3.6% (121). The reasons for failure of cancer vaccines include loss of target antigen presentation by the tumor (20) or vaccine-induced expansion of immunosuppressive cells in the host (122).

Compared to other vaccination regimens, the DC-based vaccines have among the highest objective response rates (119). Sipuleucel-T, a DC-based prostate cancer vaccine, became the
first FDA-approved therapeutic cancer vaccine following a phase III trial that showed an increased overall survival of 4.1 months compared to placebo (123). Vaccination with Sipuleucel-T involves three courses of the following protocol: isolation of autologous APCs from the patient’s blood; incubation of the APCs with a prostate tumor antigen (prostatic acid phosphatase, PAP) fused to GM-CSF to promote APC maturation; one to two days later the matured, antigen-loaded APCs are re-infused into the cancer patient. While Sipuleucel-T successfully induced detectable PAP-specific T cells in a majority of vaccinated patients (124), only one objective response was observed out of 341 vaccinated patients (123), suggesting the PAP-specific T cells may be unable to overcome local tumor immunosuppression. Despite the relatively low objective response rates in clinical trials, cancer vaccination remains an important field of investigation in cancer immunotherapy. Further refinement of target antigens and vaccination protocols may yield better therapeutic outcomes, in particular by incorporating strategies, such as irradiation, to modulate the immunosuppressive TME. An understanding of the underlying mechanisms of cancer vaccines can be translated to other fields of cancer immunotherapy.

Immune-modulating cytokines

IL-2

As described previously, IL-2 was originally discovered as a soluble “T cell growth factor” produced by activated lymphocytes (75). In 1983, the Il2 gene was cloned (125) and expressed in E. coli (126), making recombinant IL-2 available for mouse and human studies (127). In vivo, IL-2 is primarily produced by CD4⁺ T cells following activation. It signals to activated T cells and NK cells expressing the low-affinity heterodimeric IL-2 receptor (comprised of the common γ-chain and the shared IL-2/IL-15 receptor β-chain) and to CD4⁺ T_{REG} cells
expressing the trimeric high-affinity IL-2 receptor (comprised of the \( \gamma \)-chain, \( \beta \)-chain, and IL-2 receptor \( \alpha \)-chain). IL-2 has a dichotomous influence on anti-tumor T cell responses, as it can promote the proliferation and effector differentiation of anti-tumor T cells and NK cells as well as serve as a regulatory cytokine by promoting activation-induced cell death in effector cells and promoting the survival of immunosuppressive T\(_{\text{REG}}\) cells (128).

Pre-clinical studies showed that treatment of tumor-bearing mice with high doses of IL-2 resulted in regression of established tumors in association with enhanced cytotoxicity of spleen cells (129). Initial clinical trials treated a small cohort of patients with various metastatic cancers and observed objective regressions of metastatic melanoma and metastatic renal cell carcinoma using high-dose IL-2 therapy (130,131). Larger trials followed, focusing on patients with metastatic melanoma and metastatic renal cell carcinoma. Objective responses were observed in approximately 17% of cancer patients, half of which were complete responses (132). These results provided the first evidence that a purely immunological therapy could induce regressions of established tumors. The Food and Drug Administration (FDA) approved IL-2 therapy for the treatment of metastatic renal cancer in 1992 and metastatic melanoma in 1998.

In addition to its role in high-dose IL-2 therapy for cancer, IL-2 is also used in ACT therapy protocols \textit{in vitro} to expand tumor-reactive T cells for adoptive transfer and \textit{in vivo} to promote the survival and expansion of adoptively-transferred T cells. In this capacity, IL-2 serves as a critical component of successful ACT therapy. Interestingly, IL-2 can also have a negative influence on anti-tumor T cell responses. Pre-clinical studies have shown that prolonged expansion and differentiation of tumor-reactive T cells in IL-2 prior to ACT therapy actually impairs anti-tumor efficacy \textit{in vivo} (133). In patients receiving ACT therapy, the number of IL-2 doses administered positively correlates with the recovery of endogenous T\(_{\text{REG}}\) cells and predicts an unfavorable prognosis (134). Thus, IL-2 represents a double-edged sword that can be useful in promoting anti-tumor T cell responses but must be used with caution to avoid its suppressive
effects. Related cytokines in the common γ-chain cytokine family represent potential alternatives to IL-2, such as IL-7, IL-15, and IL-21.

**IL-7, IL-15, and IL-21**

IL-7 is produced by stromal cells and other non-marrow-derived cells, and its promotion of T cell expansion in lymphopenic mice (135) and patients (136) suggests it acts as an important regulator of T cell homeostasis(137). Importantly, administration of exogenous IL-7 to cancer patients was shown to promote the accumulation of CD4+ and CD8+ T cells but not Foxp3+ TREG cells (138). Thus IL-7 represents an attractive agent to promote ACT therapy, and although this combination has not yet been tested in cancer patients, its role in successful ACT therapy has been demonstrated in murine studies (96).

IL-15 also regulates T cell homeostasis, in particular the survival of memory T cells and NK cells. IL-15 is not a secreted cytokine but instead is bound to the surface of DCs and monocytes by the high-affinity IL-15Rα-chain and presented to lymphocytes expressing a heterodimeric receptor comprised of the common γ-chain and IL-2/IL-15Rβ-chain (128). In preclinical studies of ACT therapy, IL-15 has been shown to promote anti-tumor T cell responses (139). Specifically, in vitro expansion of tumor-reactive T cells in the presence of IL-15 improved the efficacy of the adoptively-transferred T cells following ACT. Additionally, both endogenous and exogenous IL-15 supported ACT-mediated tumor regressions in vivo. Preliminary results from an ongoing clinical trial in which metastatic melanoma and renal cancer patients received bolus infusions of IL-15 have shown dramatic impact on NK cell and memory CD8+ T cell accumulation, as well as regression of some metastatic lesions (140). Of note, a clinical trial (NCT01369888) was initiated to administer IL-15 in combination with ACT-TIL therapy but was
terminated early due to autoimmune toxicity. Nevertheless, IL-15 remains a promising potential adjunct for ACT therapy.

IL-21 is produced by activated CD4+ T cells and promotes the proliferation and cytotoxicity of CD8+ T cells as well as NK cells. Studies in mouse tumor models have shown that the administration of exogenous IL-21 promotes the anti-tumor efficacy of endogenous and adoptively-transferred CD8+ T cells (141). Multiple trials have shown that systemic IL-21 administration is well-tolerated and can induce objective responses in metastatic melanoma and metastatic renal cancer patients (142,143). IL-21 may be a leading candidate for inclusion with ACT therapy, as it has been shown in mouse models to improve the efficacy of adoptively-transferred T cells expanded in culture with IL-21 (144).

**IL-12**

IL-12 is primarily secreted by APCs. Administration of exogenous IL-12 to tumor-bearing mice promotes anti-tumor immunity that is mediated by CD8+ T cells (145). Interestingly, administration of IL-12 either systemically or locally at the tumor site was sufficient to induce tumor regression, suggesting a critical role for IL-12 in the modulation of the tumor microenvironment (TME). This role is further supported by a separate study that showed the anti-tumor effect of IL-12 was dependent on expression of the IL-12R by bone marrow-derived host cells but not adoptively-transferred T cells (146). T cell infiltration of the tumor resulted in an IL-12-dependent conversion of the immunosuppressive TME to a more favorable immune environment. Thus, IL-12 may be a critically important factor in the treatment of solid tumors with associated immunosuppressive TMEs.

IL-12 can be safely administered to cancer patients, although the therapeutic index is narrow and toxicity is common (147). Many recent and ongoing clinical trials are investigating
the use of IL-12 in cancer patients using a variety of delivery methods, from injection of IL-12-encoding vectors into the tumor to combination with cancer vaccines (147). One particular trial deserves mention here in which the investigators engineered autologous TIL for ACT therapy to produce IL-12 following TCR engagement (148). The IL-12-producing TIL were potent effectors, but responses and T cell persistence were short-lived. Notably, one patient who had previously failed conventional ACT-TIL therapy experienced an objective response to ACT-TIL-IL-12 therapy, demonstrating the utility of this approach in treating particularly resistant cancers.

**Immune-modulating antibodies**

**Anti-CD40**

Agonists of the CD40 receptor can induce the amplification of an anti-tumor immune response. Anti-CD40 agonist antibody is an innate triggering agent that licenses DCs to become potent T cell-activators. Under non-inflammatory conditions, unlicensed (or “immature”) dendritic cells do not express costimulatory molecules and thereby promote peripheral tolerance by inducing T cell anergy (149). Anergic T cells will not respond or become activated following subsequent antigen encounters. This mechanism of peripheral tolerance limits the activation of autoreactive T cells that may escape thymic negative selection; it also limits the activation of tumor-reactive T cells that recognize tumor antigens that may be self-antigens. To initiate a T cell response, DCs must become licensed (“mature”) to express costimulatory molecules and inflammatory cytokines, resulting in activation of antigen-specific T cells (150). This licensing is mediated by the CD40 receptor, expressed by DCs and other pAPCs (151). Activated CD4⁺ T helper cells express CD40 ligand (CD40L) and are able to license DCs by engaging the CD40
receptor. Thus, the administration of anti-CD40 agonist antibody can bypass the need for CD4^+ T cell help and promote the activation of tumor-specific CD8^+ T cells (Fig. 2-8).

Pre-clinical studies in mice have shown that treatment with agonist anti-CD40 antibodies can promote endogenous anti-tumor CD8^+ T cell responses in vivo (152), but may also promote deletion of the responding T cells unless additional agents are administered in combination with anti-CD40. Furthermore, anti-CD40 can promote tumor regression in a T cell-independent manner through activation of host macrophages (153). Anti-CD40 is also effective in promoting the in vivo expansion and differentiation of donor T cells when used in combination with ACT therapy (154-157), although this therapeutic combination has not been investigated in human patients. In the current study, we investigate the use of anti-CD40 as a host-conditioning regimen for ACT therapy.

In clinical trials, anti-CD40 has been used as a single agent to treat patients with a variety of cancers and has been shown to induce tumor-specific T cell responses and to mediate objective responses, albeit only in patients with metastatic melanoma (158). Follow-up trials using anti-CD40 in combination with chemotherapy achieved objective responses in patients with a variety of cancers (159,160).

**Anti-CTLA4**

The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an inhibitory receptor, expressed by activated and regulatory T cells, critical in preventing lymphoproliferative disease and autoimmunity (161). CTLA-4 binds the B7 costimulatory ligands with a 100- to 1000-fold higher affinity compared to the costimulatory receptor CD28 and can inhibit effector T cell activity by outcompeting CD28 for B7 ligands (38). Additionally, CTLA-4 down-regulates
immune responses by promoting T\textsubscript{REG} cell inhibitory functions and tempering immune-stimulating APCs (162).

Experimental studies in mouse tumor models have shown that therapy with anti-CTLA-4 antagonist antibodies can promote control of multiple tumor types (163). Furthermore, this therapy could be extended to less responsive tumors by combining anti-CTLA-4 with tumor antigen vaccination to enhance immunogenicity (164,165). Interestingly, anti-CTLA-4-mediated tumor regression was reliant on CD8\textsuperscript{+}, but not CD4\textsuperscript{+} T cells (164), suggesting the primary mechanism of anti-CTLA-4 therapy was the potentiation of CD8\textsuperscript{+} T cell effector responses rather than inhibition of T\textsubscript{REG} cell-mediated immunosuppression (Fig. 2-9). In addition to APCs, tumor cells can also express B7 molecules (166). Thus, the mechanism of anti-CTLA-4 therapy may involve inhibition of B7/CTLA-4 engagement in the TME.

In 2010, an anti-CTLA-4 antagonist antibody, Ipilimumab, gained FDA-approval for the treatment of metastatic melanoma following a phase III clinical trial that showed a 3.5 month improvement in overall survival (167). In this trial, the objective response rate was approximately 10\% and the complete response rate was 0.6\% among patients treated with Ipilimumab. Interestingly, the combination of Ipilimumab with IL-2 therapy in a follow-up trial improved the objective and complete response rates to 25\% and 17\%, respectively (168). Collective consideration of the results of pre-clinical and clinical studies suggests that anti-CTLA-4 therapy acts to potentiate anti-tumor T cell responses in cancer patients by inhibiting immune- and tumor-mediated negative feedback, and that the use of anti-CTLA-4 in combination with additional immunotherapies, such as those that promote the expansion of anti-tumor T cells, may improve therapeutic outcomes.

It is important to note that anti-CTLA-4 therapy is associated with numerous and sometimes severe autoimmune side effects. In a large clinical trial (167), 60\% of the patients treated with ipilimumab experienced at least one immune-related adverse event; the most
common immune-related adverse effect was diarrhea, which was reported in approximately 30% of the patients treated with ipilimumab. Additional adverse events included severe colitis, which required treatment with anti-TNFα antibody (infliximab), and inflammation of the pituitary, which required hormone replacement therapy. Notably, 2.1% of treated patients died due to treatment-related causes. Thus, the use of anti-CTLA-4 as a single agent or as part of a therapeutic combination must be weighed against the risks of severe toxicity, which has been observed to persist for years following treatment.

Anti-PD-1/anti-PD-L1

PD-1 is an inhibitory receptor expressed upon activation of T cells (44). Ligation with PD-L1 inhibits T cell proliferation and effector function (45). PD-L1 is expressed by human tumors (50,51); this expression has been shown experimentally to occur in response to T cell infiltration (51). In pre-clinical mouse models, expression of PD-L1 by tumor cells directly inhibited effector T cell cytotoxicity, and therapeutic blockade of PD-1 signaling with antibody antagonists to PD-1 or PD-L1 promoted endogenous anti-tumor T cell responses (Fig. 2-10) (169,170). Initial clinical trials using anti-PD-1 therapy achieved objective regressions in patients with melanoma, renal cancer, and lung cancers (171,172). Interestingly, there were no objective responses to anti-PD-1 therapy in patients bearing PD-L1+ tumors (172), suggesting the mechanism of anti-PD-1 therapy is localized to the tumor site. Follow-up trials have shown consistent success in treating larger cohorts of patients with a variety of cancers using anti-PD-1 and anti-PD-L1 therapies (173). In 2014, both Pembrolizumab and Nivolumab (anti-PD-1 antibodies) were FDA-approved to treat metastatic melanoma and non-small cell lung carcinoma, respectively. Recent clinical trials using these antibodies have achieved objective response rates of 26% (174) and 40% (175), respectively.
The prospect of combination immunotherapies

The immunotherapies described above affect the anti-tumor T cell response at multiple stages. Logical combinations of non-redundant immunotherapies can be expected to show additive or synergistic effects. For example, anti-CTLA-4 and anti-PD-1 represent a logical combination, as the former targets T cell inhibition by T\textsubscript{REG} cells and the latter targets T cell inhibition by the tumor. Theoretically, release of the two inhibitory signals concurrently should promote a stronger T cell response than either agent alone. Indeed, this combination has been investigated in metastatic melanoma patients shown to improve objective and complete response rates without increasing side effects (176). As mentioned, the combination of IL-2 therapy with anti-CTLA-4 therapy has also been shown to improve response rates higher than either agent alone (168). This combination demonstrates a logical combination of a checkpoint inhibitor with a stimulatory antibody, effectively “releasing the brakes” and “stepping on the accelerator” at the same time. Other potential combinations could replicate this effect, such as the combination of stimulatory anti-CD40 with a checkpoint inhibitor such as anti-CTLA-4 or anti-PD-1.

One potentially interesting advantage to ACT therapy may be that tumor-reactive T cells are available \textit{ex vivo} for therapeutic manipulation. These T cells could be treated with a checkpoint inhibitor prior to re-infusion, allowing the antibody to be used at much lower doses and without potential off-target effects. Checkpoint inhibitors could be used to increase effector T cell function (anti-PD-1) or to neutralize any T\textsubscript{REG} cells (anti-CTLA-4) contaminating the re-infusion product. Following ACT, combination immunotherapies could be administered, such as anti-CD40, to promote anti-tumor T cell activity \textit{in vivo}. In the current study, we investigate the prospect of utilizing anti-CD40 in combination with ACT therapy as a host-conditioning regimen to promote \textit{in vivo} donor T cell activation and accumulation.
In summary, numerous immunotherapies are under investigation in clinical and preclinical studies. The recent influx of FDA-approved cancer immunotherapies into mainstream medicine may represent the emergence of a revolution in the philosophy and practice of cancer treatment that began many years ago.

Radiation therapy and cancer

As γ-radiation passes through a living cell, it induces DNA damage that, when severe, results in cellular apoptosis. For many years, the primary mechanism of action of radiation therapy of cancer was believed to be direct cytotoxicity to tumor cells. More recently, the immune response to radiation has been shown to play a critical role in the therapeutic benefit of radiation therapy of cancer (177). Here, the immunological corollaries of tumor irradiation are described, as the impact of radiation on the tumor and local TME is an important consequence of WBI conditioning for ACT therapy investigated in the current study.

The immune response following radiation therapy

In rare occurrences, the local irradiation of a tumor can result in regression of a distant tumor outside the field of irradiation. In 1953, R.H. Mole coined the term “abscopal effect” to describe this phenomenon of distant regression of tumors (e.g. regression of metastases) following local irradiation (178). The mechanisms that mediated the abscopal effect remained largely undefined until 2004, when Demaria et al. showed experimentally that the abscopal effect is T cell-mediated. The investigators induced two subcutaneous tumors in mice, one in each flank. In immune-competent mice treated with Flt3 ligand (to activate DCs), local irradiation of only one tumor induced regression in the unirradiated contralateral tumor (179). This abscopal
effect was lost when the same experiment was performed in immune-deficient nude mice, demonstrating the critical role of endogenous T cell immunity in mediating regression of the unirradiated tumor. Furthermore, this study provided evidence that tumor irradiation was capable of inducing anti-tumor immunity.

More recently, immune activity has been shown to be a critical component to successful radiation therapy. Using the B16 melanoma model, Burnette et al. demonstrated the importance of type I IFN production following local irradiation (180). The investigators showed that the therapeutic effect of local irradiation was lost in mice insensitive to type I IFN due to genetic knockout of the type I IFN receptor. Furthermore, the target of type I IFN was not the tumor cells but the host immune cells, demonstrating a requirement for immune activation in the therapeutic response to local irradiation. Recently, a pair of studies by Deng et al. and Woo et al. collectively demonstrated a more complete mechanism to explain the induction of type I IFN production following local irradiation. Deng et al. showed that the induction of type I IFN production by DCs following exposure to irradiated tumor cells was mediated by signaling through the stimulator of IFN genes (STING) in DCs (181). Furthermore, it was demonstrated by Woo et al. that DCs captured tumor-derived DNA at the tumor site, resulting in STING activation and type I IFN production (182). These studies have implicated that local irradiation-induced tumor cell damage leads to DC detection of tumor-derived DNA, the induction of type I IFN production, and the activation of innate and adaptive immunity. Collectively, these findings define a critical role for the immune response in the success of local radiation therapy and suggest these mechanisms can be exploited for the benefit of cancer immunotherapy.

**Effects of irradiation on the TME that support anti-tumor immunity**

In a healthy host, normal cellular turnover results in the apoptosis of billions of cells each
day, which are removed by phagocytosis in an immunologically silent manner. Recently, an alternative apoptotic mechanism has been identified in which the apoptosis of host cells can result in an immune response. This process, which can be induced by \( \gamma \)-irradiation and select chemotherapies, is called immunogenic cell death (ICD) (183). Two underlying mechanisms of ICD have been described. First, tumor cells undergoing ICD translocate the protein calreticulin, which typically resides at the endoplasmic reticulum, to be exposed at the cell surface. \( \gamma \)-radiation has been shown to promote calreticulin translocation by tumor cells (184). Exposure of calreticulin promotes phagocytosis by DCs and therefore uptake of tumor antigens (185). Second, tumor cells undergoing ICD also release cellular danger signals, which bind toll-like receptors on DCs and result in DC activation. One example of a well-characterized cellular danger signal is the cytoplasmic protein high mobility group box 1 (HMGB-1). HMGB-1 is a multi-functional protein. In the cell nucleus, HMGB-1 binds DNA to promote transcription factor binding. HMGB-1 can be released into the extracellular environment via passive secretion from apoptotic and/or necrotic cells. In the extracellular environment, HMGB-1 has been shown to bind to toll-like receptor 4 (TLR4) on the surface of DCs, invoking the DCs to up-regulate antigen presentation and activate naive T cells (186). Thus, the induction of ICD is one mechanism by which irradiation can promote anti-tumor immunity.

Irradiation of a tumor has other important immune effects. Irradiation of tumor cells has important effects on antigen-presentation, such as up-regulation of MHC class I molecule expression (187). Irradiation also increases the degradation of cellular proteins and up-regulates the expression of novel proteins; both of these mechanisms contribute to increased diversity in the peptide repertoire and potentially new immune targets (188). Furthermore, tumor cells have been shown to up-regulate surface expression of the death receptor Fas following irradiation, rendering these cells more susceptible to immune-mediated elimination by FasL-expressing T cells (189).
Local irradiation has been shown to enhance the accumulation of tumor-reactive T cells in the tumor-draining lymph node (98), and can also increase recruitment of immune effector cells to the TME by inducing local chemokine production (190) and expression of vascular cell adhesion molecule-1 (VCAM-1) by tumor vasculature (98,191). A recent elegant study by Lim et al. demonstrated that chemokine expression, T cell infiltration, and T cell effector function at the TME were all mediated by local irradiation-induced type I IFN production (192). Collectively, these studies suggest that local irradiation of the tumor has a profound effect on the TME and may render a previously immunosuppressive TME more permissive to anti-tumor T cell accumulation and tumor regression.

**Local irradiation and immunotherapy**

The effects of irradiation on the TME and responding immune cells described in this section suggest that synergy between local irradiation therapy and immunotherapy is likely. Preclinical studies have demonstrated an enhanced therapeutic effect by combining local irradiation with multiple immunotherapies (193), including anti-PD-1 (194) and anti-CTLA-4 (195). Interestingly, the use of anti-PD-1 or anti-CTLA-4 alone had no impact on survival in these models compared to untreated animals, however, when combined with local irradiation, synergism of anti-PD-1 and anti-CTLA-4 with local irradiation resulted in significant extension in survival. These results demonstrate the ability of local irradiation to promote an anti-tumor immune response that was otherwise completely ineffective.

The investigation of combination therapies has also yielded promising results in human trials. A combination of local irradiation with IL-2 therapy resulted in response rates above 50% in patients with metastatic melanoma and renal cell carcinoma (196). A 12.5% response rate was achieved combining local irradiation with a dendritic cell vaccine for liver cancer (197). Notably,
a study published during the preparation of this dissertation provided strong data to support the combination of local irradiation with checkpoint inhibitors (198). The investigators demonstrated significant tumor regressions using a combination of local irradiation and anti-CTLA-4 in melanoma patients. Furthermore, in a mouse model of melanoma, the authors demonstrate that a prominent resistance mechanism to this combination therapy is expression of PD-L1 at the tumor site, thus providing support for additional trials investigating the three-part combination therapy of local irradiation, anti-CTLA-4, and anti-PD-1. A trial combining local irradiation with ACT therapy in five patients did not show any evidence of response, however, given the low number of patients, strong conclusions regarding the potential for this combination therapy cannot be made (199).

Collectively, clinical and preclinical evidence suggest that the combination of local irradiation with immunotherapies is a rational approach to improving response rates in cancer patients. Many questions remain, including: whether the use of local irradiation can improve the durability of anti-tumor responses; and whether the use of local irradiation will promote the success of immunotherapies in less immunogenic cancers. In the current study, we investigate the impact of local and systemic irradiation on ACT therapy to determine whether irradiation-induced changes to the TME are required for ACT-mediated tumor regression and long-term tumor control.

**Experimental mouse tumor models and immunotherapy**

**Overview**

Mouse models of cancer are a heterogeneous collection of experimental systems designed to induce tumor formation in mice for scientific investigation of cancer biology and therapeutics.
These models provide an important platform for investigation of biological mechanisms \textit{in vivo}, as well as translating bench-top research into clinical trials. Tumors can be generated from exogenous tumor cells or from induced transformation of host cells (200). Transplantable tumor models require injection of tumor cells to induce tumor formation. Typically, tumor cells are injected subcutaneously, which allows the investigator to visually monitor and measure tumor growth. Transplantable tumor models provide investigators with a convenient method of generating tumors of consistent and measurable size, as well as the ability to easily vary the tumor cell type (e.g. B16 melanoma cell versus Renca renal cancer cell) and the genetic background of the host (e.g. wild-type and genetic-knockout mouse strains). Indeed, much of our understanding of tumor immunology and immunotherapy results from studies performed using transplantable tumor models. Transplantable tumors grow quickly, however, and do not necessarily provide the most accurate model of human tumors, which arise more slowly and with a well-developed stroma. Notably, responses to experimental immunotherapies have been shown to be reduced when tumor cells are injected orthotopically into their natural tissue of origin (e.g. pancreatic cancer cells into pancreas) compared to subcutaneous injection (201). This effect was mediated by a more suppressive TME in orthotopic tumors, and it highlights the value of tumor models that more realistically replicate human tumors and TMEs.

Spontaneous tumor models induce tumor formation using carcinogenic agents or genetic engineering, such as the introduction of an oncogene or the deletion of a tumor suppressor gene. Spontaneous tumor models generate tumors of autochthonous origin, i.e. tumors developing from the host tissue of origin. Autochthonous tumors arise in conjunction with a well-developed tumor stroma and microenvironment that more closely mimics human disease (200).

In transgenic autochthonous tumor models, the expression of a transgenic oncogene induces a state of cellular genetic instability in which cells are highly susceptible to genetic mutation; this instability leads to reproducible neoplastic transformation and tumor development.
Because autochthonous tumors develop in an immune-competent host and are seen as “self”, they can induce immunological tolerance similar to human tumors (202). Autochthonous tumor models can be engineered to direct tumor formation to a particular tissue using tissue-specific promoters. An example of an autochthonous tumor model that utilizes a tissue-specific promoter is the RIP-Tag (rat insulin promoter-SV40 T antigen) model, in which oncogenic T Ag expression is driven by the insulin promoter in the β-cells of the pancreas and leads to the formation of pancreatic β-cell tumors (203). Because autochthonous tumors grow more slowly than implantable tumors, the cost is considerably higher. Additionally, the “spontaneous” nature of tumor growth in these models means all mice of a given age will not have the same size or stage tumors, which can lead to higher variability in experimental results.

The studies presented in this dissertation were performed using a transgenic tumor model in which expression of the simian virus 40 (SV40) large T antigen oncoprotein in the choroid plexus of the brain induces autochthonous tumor formation in line SV11 mice (204). The SV11 model provides an excellent system for the investigation of T cell immunotherapy of cancer because it induces tumor formation using an oncoprotein that also serves as a tumor antigen with defined T cell epitopes (205). Additionally, the autochthonous nature of the SV11 tumor mimics the common clinical scenario in which tumors are detected at an advanced stage with well-established tumor stroma and microenvironment.

SV11 mouse model of T antigen-induced choroid plexus tumors

SV40 large T antigen: from monkey to mouse

SV40 (simian virus 40) is an oncogenic virus of the Polyomaviridae family. Upon its original characterization, the “vacuolating virus”, named for the numerous cytoplasmic vacuoles
observed in infected monkey kidney cells, was designated SV40 by Sweet and Hilleman in 1960. At the time of SV40 discovery, rhesus monkey kidney cells were being used for large-scale production of the inactivated polio vaccine (IPV) developed by Jonas Salk. Batches of the IPV used between 1955 and 1963 were found to be contaminated with SV40, exposing tens of millions of Americans to the virus. The cancer-causing potential of SV40 in humans remains a controversial subject, although large-scale retrospective studies have found no increase in cancer incidence among patients that received contaminated IPV (206,207).

The SV40 genome encodes a total of six viral proteins – three structural and three non-structural proteins. Two of the non-structural proteins, called large tumor antigen (T Ag) and small tumor antigen (t Ag), perform functions that support the viral life cycle. T Ag initiates DNA replication by binding the origin of replication on the viral genome, provides ATP-dependent helicase activity necessary for DNA-unwinding during genome replication, and activates the late-gene viral promoter (208). T Ag is not absolutely required for viral replication, but its presence increases early viral promoter activity (209). Most notably, both T Ag and t Ag promote neoplastic transformation, each through distinct mechanisms. T Ag promotes cell cycle entry by binding and sequestering tumor-suppressor proteins p53 (210,211) and pRb (212,213). T Ag enhances neoplastic transformation by binding regulatory protein pp2A to promote cell proliferation (214,215). Expression of T Ag alone is sufficient to promote tumorigenesis in transgenic mice (216). These tumors develop in the choroid plexus of the brain in association with high-level T Ag expression under the control of the natural SV40 viral promoter. This promoter has been shown to direct the tissue-specific expression of T Ag in transgenic mice (217).
Background and pathology of the SV11 mouse line

The SV11 mouse line was established by microinjection of the pSV11 plasmid containing the SV40 promoter and T Ag sequence into C57BL/6 (B6) mouse embryos (218). Germline integration of the pSV11 plasmid resulted in development of choroid plexus brain tumors in adult mice (216,218,219). Following its establishment, the SV11 mouse line was well characterized in 1987 by Van Dyke and colleagues (204). As mentioned, SV11 mice develop tumors of the choroid plexus, an epithelial tissue located within the brain ventricles and responsible for secretion of the cerebrospinal fluid (CSF) that surrounds the brain and spinal cord. Van Dyke and colleagues first observed detectable T Ag expression at 2 weeks of age using Western blot analysis of brain lysates. The first histopathological changes were noted at approximately 40 days of age with the appearance of grade I microscopic foci of anaplastic choroid plexus cells with enlarged, darkly staining nuclei and grade II microscopic papillomas. Low-level T Ag expression continued until roughly 80 days of age, at which time T Ag levels increased dramatically. In association with the increase in T Ag expression, choroid plexus tumors progressed to grade III macroscopic papillomas and grade IV carcinomas, becoming lethal at a mean of 104 days of age. Choroid plexus tumors were observed in all SV11 mice, demonstrating the high penetrance of this phenotype. Tumor metastases were rare and were restricted to the central nervous system (CNS).

In addition to the choroid plexus, low levels of T Ag protein have been detected in the kidney of SV11 mice (219), and T Ag mRNA transcripts have been detected in the thymus (unpublished observation, T. D. Schell). Despite low-level expression in these tissues, T Ag-induced tumor formation in SV11 mice is restricted to the choroid plexus. As will be described in the next section, SV11 mice are immunologically tolerant to T Ag. T Ag expression in the thymus may be responsible for this tolerance, though this has not been formally proven. The
immunological consequences of T Ag expression in the kidneys of SV11 mice have not been investigated previously and may also contribute to tolerance, similar to human cancers.

**CD8\(^+\) T cell recognition of T Ag**

Immunization of *H-2\(^b\)* B6 mice with T Ag-expressing syngeneic *H-2\(^b\)* cell lines induces a CD8\(^+\) cytotoxic T cell response directed against T Ag (220). In SV11 mice (*H-2\(^b\)*), choroid plexus tumor cells express high levels of T Ag, making this protein an ideal target for cytotoxic immunotherapy (204). Four CD8\(^+\) T cell determinants have been identified within T Ag and named as follows: site I, site II/III, and site V (*H-2D\(^b\)*-restricted); and site IV (*H-2K\(^b\)*-restricted) (Fig. 2-11) (221-225). Immunization of naïve B6 mice with T Ag-expressing cell lines results in detectable CD8\(^+\) T cell responses to sites IV, I, and II/III, in decreasing magnitude, respectively, but not to site V (226). Priming of site V-specific naïve T cells is only directly detectable following immunization of B6 mice in the absence of the immunodominant determinants, such as with cell lines expressing T Ag variants in which the immunodominant determinants have been mutated or deleted (226). Thus, site V has been characterized as immunorecessive.

In contrast to B6 mice, CD8\(^+\) T cell priming to the immunodominant determinants is not detectable in SV11 mice following immunization with T Ag. This functional tolerance is likely the result of T Ag expression in the thymus and its recognition as a self-antigen, leading to clonal deletion during T cell development – termed central tolerance. Tolerance to T Ag in SV11 mice allows T Ag-expressing choroid plexus tumors to grow in the absence of a detectable endogenous anti-tumor immune response to the known T cell epitopes.
Studies of ACT therapy in the SV11 mouse model

Recognition of T Ag as a self-antigen in SV11 mice results in immunological tolerance and the inability to respond to T Ag immunization. While strong, highly-directed immunization strategies can prime endogenous CTLs specific for the immunorecessive site V epitope (but not the immunodominant epitopes) in SV11 mice, strategies targeting endogenous site V CTLs have shown no therapeutic benefit (227). In this section, previous studies investigating ACT therapy in SV11 mice will be presented that have collectively shown that immunological tolerance in SV11 mice can be overcome using ACT of lymphocytes from syngeneic donor mice, and that ACT-based approaches can have significant therapeutic effects. In these studies, both wild-type B6 mice and T cell receptor (TCR)-transgenic mice were used as donors for ACT therapy. Naïve B6 mice provide a source of polyclonal T cells with a relatively low frequency of T Ag-reactive CTL precursors, which can be increased by T Ag immunization of B6 donor mice. TCR-transgenic mice express a transgenic TCR α chain and β chain pair in CD8+ T cells, resulting in a high frequency of naïve T cells that recognize a specific determinant. Three lines of TCR-transgenic mice were used in the studies presented in this section: line F2025 mice express a transgenic TCR specific for the H-2Kb-restricted site IV epitope of T Ag; line 459 mice express a transgenic TCR specific for the H-2Db-restricted site V epitope of T Ag; and line 416 mice express a transgenic TCR specific for the H-2Db-restricted site I epitope of T Ag. Tumor-reactive CTL were primed in vivo, either by tumor antigen-immunization of B6 donor mice prior to ACT or by ACT of naïve donor cells from B6 or TCR-transgenic mice followed by in vivo priming in SV11 mice mediated by immunization or the endogenous tumor antigen. Specifically, these studies show that WBI conditioning prior to ACT in SV11 mice promotes donor T cell priming by the endogenous tumor antigen and the long-term persistence of T Ag-specific donor T cells, and that epitope IV-specific
T cells are sufficient to mediate long-term survival of WBI-conditioned SV11 mice. These studies will be presented in detail here.

A 1999 study by Schell et al. showed that SV11 mice were functionally tolerant to T Ag, but that T Ag-specific CTLs could be primed in SV11 mice that received ACT of B6 splenocytes (202). While immunization of naïve B6 mice with recombinant vaccinia virus expressing full-length T Ag (VV-941T) induced robust priming of endogenous CTLs specific for the immunodominant epitopes of T Ag, SV11 mice were unable to generate endogenous CTL responses following similar immunization. Adoptive transfer of naïve B6 splenocytes into 40 day-old SV11 mice (bearing microscopic tumors) followed by immunization with VV-941T induced priming of epitope IV-specific CTLs, and prolonged median survival from 105 days (untreated) to 149 days. The administration of low-dose WBI prior to ACT of naïve B6 splenocytes induced epitope IV-specific CTL priming by the endogenous tumor antigen. This treatment significantly increased median survival to 249 days. Collectively, these results demonstrate that the functional tolerance of SV11 mice to T Ag can be overcome by ACT of naïve B6 splenocytes. Priming of the transferred donor cells was epitope IV-specific and required adjuvant immunization or WBI conditioning, which was associated with tumor control in SV11 mice.

In a follow-up study, SV11 mice with more advanced tumors were treated at 80 days of age (228). Following WBI plus ACT with naïve B6 splenocytes, functional T Ag-specific CTLs primed by the endogenous tumor antigen persisted as late as 70 days post-ACT. Interestingly, analysis at 20 and 30 days post-ACT detected only epitope IV-specific CTLs, but by 70 days post-ACT both epitope I- and epitope IV-specific CTLs were detected, suggesting tumor control is mediated by CTLs specific for multiple tumor epitopes at later time points. Survival of SV11 mice following this treatment was extended further by combining WBI conditioning with ACT of splenocytes from B6 mice that were immunized with full-length T Ag-expressing WT-19 cells (T
Ag-immune splenocytes). In contrast to ACT with naïve B6 splenocytes, functional analysis following ACT with T Ag-immune splenocytes at 20, 30, and 70 days post-ACT into WBI-conditioned SV11 mice detected CTLs specific for epitopes I, II/III, and IV in both spleen and brain. Thus, the increase in survival mediated by T Ag-immune donor cells may result from the increased T Ag-specific CTL precursor frequency in the donor cell pool and/or the targeting of multiple epitopes. The contribution of epitope I- and II/III-specific CTLs to prolonging SV11 survival was confirmed in a later study (229). In summary, the 2001 study by Schell et al. demonstrates epitope IV-specific CTLs primed by the endogenous tumor antigen in WBI-conditioned SV11 mice mediated durable tumor control by persisting long-term at the tumor site and in the peripheral lymphoid organs. Additionally, the use of T Ag-immune donor cells targeting multiple T Ag epitopes further prolonged survival of SV11 mice.

A study by Yorty et al. further investigated the differences in donor T cell accumulation following ACT of naïve versus T Ag-immune donor cells into WBI-conditioned SV11 mice at 80 days of age (229). The authors found that epitope IV-specific donor T cells accumulated in the brain at a high-level as early as five days post-ACT with T Ag-immune donor cells and were maintained beyond 40 days post-ACT. In contrast, low-level accumulation in the brain was first detected seven days post-ACT with naïve donor cells and continued to increase until three to four weeks post-ACT when accumulation of naïve and T Ag-immune donor cells was equivalent. Similarly, accumulation of epitope IV-specific T cells in the spleen was accelerated in mice receiving T Ag-immune donor cells versus naïve donor cells. However, by 40 days post-ACT both groups maintained a similar frequency of epitope IV-specific T cells in brain and spleen. These results indicated that transfer of T Ag-immune donor cells correlated with more rapid T-cell accumulation in both brain and spleen. Additionally, the authors showed that functional epitope I- and IV-specific T cells persisted beyond 170 days post-ACT with T Ag-immune donor cells. A 10-fold reduction in dose of T Ag-immune donor cells resulted in reduced epitope IV-
specific T-cell frequency in the brain at ten days but not 30 days post-ACT. This lag in T-cell accumulation was associated with reduced tumor control. Finally, the authors showed that ACT with immune donor cells from B6 mice immunized with a site IV-only expressing cell line prolonged median survival in WBI-conditioned SV11 mice to 226 days. In summary, this study showed that control of tumor burden in WBI-conditioned SV11 mice is associated with early, high-level accumulation of site IV-specific T cells in the brain that persist long-term. Additionally, maximum early T cell accumulation and tumor control were achieved using the highest dose of donor cells. Finally, epitope IV-immune donor cells were sufficient to promote extended survival of WBI-conditioned SV11 mice.

Tatum and colleagues investigated the role of site IV-specific T cells in control of SV11 tumors (117). First, the authors showed that naïve donor cells devoid of site IV-specific T cells, isolated from spleens of site IV-tolerant line 243 mice, were unable to prolong survival of WBI-conditioned SV11 mice. This result indicated that site IV-specific T cells were required for ACT-mediated control of tumors in WBI-conditioned SV11 mice. Furthermore, ACT with donor cells comprised entirely of naïve site IV-specific T cells mediated long-term tumor control in WBI-conditioned SV11 mice. These donor cells were isolated from line F2025 TCR-transgenic mice (TCR-IV). Following ACT, donor T cells: (i) accumulated rapidly in the brains of WBI-conditioned mice by day +5 post-ACT, (ii) mediated acute tumor regression by day +10, and (iii) promoted long-term survival in association with donor T cell persistence in the lymphoid organs and at the tumor site. This WBI+ACT therapeutic protocol using TCR-IV transgenic donor T cells will be used in the studies presented in this dissertation. In summary, these results showed that site IV-specific T cells are both a necessary and sufficient component of the donor cell pool to achieve ACT-mediated tumor control in WBI-conditioned SV11 mice. Furthermore, TCR-IV donor T cells persisted beyond 95 days post-ACT in the brains and spleens of WBI-conditioned,
but not unconditioned SV11 mice, confirming that the use of transgenic T cells replicates previous findings using wild-type T cells.

Multiple studies from our research group have investigated ACT therapy of SV11 mice using transgenic donor T cells that recognize the site V immunorecessive determinant (TCR-V). It is important to note that WBI conditioning was not investigated in mice receiving TCR-V ACT. These studies demonstrate that ACT of naïve TCR-V donor T cells results in donor T cell-priming against the endogenous tumor antigen, however, these donor T cells fail to accumulate in the brain unless specific immunization to site V is included in the therapeutic protocol. Specifically, immunization given on days 0 and +7 promoted increased site V donor T cell accumulation in the brain and significant survival prolongation (230). A follow-up study showed that the timing of vaccination had a significant effect on the outcome (155). Vaccination on day +7 was required to promote significant donor T cell accumulation in the brain and significant extension of survival, compared to vaccination on day 0, which did not impact survival. These studies demonstrate that the use and timing of host-conditioning strategies such as vaccination can have a significant impact on ACT therapy by promoting enhanced donor T cell accumulation at the tumor site.

It is important to mention additional studies from our research group that investigated the combination of anti-CD40 conditioning with ACT therapy in the SV11 model and other autochthonous T Ag-induced tumor models, as these previous results have relevance to the current study. CD40 is expressed at the surface of professional antigen-presenting cells, including DCs (151). Engagement by its natural ligand CD40L (CD154), expressed by CD4+ T cells, results in licensing of the DC to become an effective activator of CD8+ T cells through up-regulation of antigen-presentation, costimulatory molecule expression, and cytokine production (Fig. 2-8) (150). The therapeutic use of agonist anti-CD40 antibody, therefore, can bypass the need for
CD4+ T cell help and enhance anti-tumor CD8+ T cell responses, including in combination with ACT therapy.

Indeed, the administration of anti-CD40 in combination with ACT of site I- or site V-specific donor T cells (TCR-I and TCR-V, respectively) promoted robust donor T cell priming against the endogenous tumor antigen (154,155,231). Administration of anti-CD40 with ACT of TCR-I donor T cells reversed T cell tolerance and promoted donor T cell accumulation, effector function, and persistence in a model of osteosarcoma (231). In a pancreatic tumor model, the combination of anti-CD40 with ACT of TCR-I donor T cells also promoted significant donor T cell accumulation and effector function, however, donor T cells were ultimately deleted (154). In SV11 mice, anti-CD40 enhanced the rapid accumulation of TCR-V donor T cells in the brain, however, the addition of tumor antigen vaccination was required to optimize donor T cell accumulation and persistence following anti-CD40+ACT therapy (155). These results demonstrate the ability of anti-CD40 to enhance donor T cell activation and accumulation when used in combination with ACT therapy, but also imply that additional therapeutic strategies, such as vaccination, may be required to promote donor T cell persistence.

Collectively, these studies demonstrate that line SV11 mice bearing T Ag-induced choroid plexus tumors are immunologically tolerant to T Ag, likely due to its recognition as a self-antigen in the thymus. This model mimics the common case of a human cancer patient whose immune system has been rendered tolerant and unable to oppose tumor growth. In this mouse model, therapeutic approaches using ACT are ineffective unless combined with immunization or WBI conditioning to overcome the immunosuppressive host environment and promote robust T cell accumulation at the tumor site. In clinical trials, WBI conditioning is used in combination with ACT to successfully treat patients with advanced cancers, including complete responses in some patients. Justification for the use of WBI as a conditioning regimen is founded in clinical studies showing increases in the frequency of responses following ACT in
patients conditioned with WBI prior to ACT (91). A thorough understanding of the underlying mechanisms of WBI conditioning that promote ACT-mediated anti-tumor responses is necessary to improve these therapies further. To this end, an investigation of ACT therapy in SV11 mice was undertaken. The content of this dissertation constitutes the culmination of this investigation on the impact of host-conditioning regimens in ACT therapy.

Specific Aims

Relevance:

Developments in cancer immunotherapies remain at the cutting edge of advancement in the treatment of cancer. Despite numerous advantages over current standard-of-care treatments, successfully achieving complete responses remains elusive and unpredictable. Host-conditioning with whole-body irradiation (WBI) prior to adoptive T cell transfer (ACT) therapy has been shown to increase response rates, including complete responses. To improve these therapeutic protocols and response rates, the underlying mechanisms by which host-conditioning promotes ACT-mediated complete responses must be understood, and this represents a gap in the knowledge.

In a mouse model of T antigen-induced brain cancer (SV11), we have previously shown that ACT following sub-lethal (4 Gy) lymphodepleting WBI promotes regression of established tumors and long-term survival (117). Tumor regression and long-term survival has been associated with: (i) a rapid, high level accumulation of donor T cells at the tumor site by day +5 post-ACT; and (ii) the persistence of functional donor T cells at the tumor site as late as day +135 post-ACT. The objective of this project is to define the mechanisms of WBI conditioning required for successful ACT therapy of established autochthonous brain tumors.
**Hypothesis #1:** The local effects of WBI conditioning on the tumor microenvironment induce a brief window of opportunity for successful ACT therapy.

**Specific Aim 1:** Define the window of opportunity following WBI conditioning during which ACT therapy can successfully mediate tumor regression and long-term survival

Previous results indicated that donor T cells accumulate rapidly in the brain following WBI conditioning (117). Here, our goal was to determine whether delaying donor T cell accumulation following WBI would compromise tumor control. To test this, we delay ACT at varying intervals following WBI conditioning and assess donor T cell accumulation, acute tumor regression, and overall survival.

**Specific Aim 2:** Determine the requirement for the local and systemic effects of irradiation to promote ACT-mediated tumor regression and long-term survival

Both local and systemic effects of irradiation have been shown to contribute to donor T cell accumulation and the success of ACT therapy (96,232). We sought to determine whether irradiation localized to the head or to the body was sufficient to promote donor T cell accumulation in the brain, acute tumor regression, and long-term survival.

**Hypothesis #2:** Rapid, high-level donor T cell accumulation and persistence at the tumor site is critical to promote successful ACT therapy.

**Specific Aim 1:** Determine the requirement for rapid, high-level donor T cell accumulation at the tumor site to promote ACT-mediated tumor regression and long-term survival

Previous results demonstrated that donor T cells accumulate rapidly at the tumor site in irradiated but not in unirradiated mice (117). Here, we asked whether rapid donor T cell
accumulation in the absence of irradiation conditioning was sufficient to promote ACT-mediated
tumor regression and long-term survival. To do this, we substitute agonist anti-CD40 antibody,
which promotes donor T cell accumulation in the brain, for WBI conditioning and assess acute
tumor regression and long-term survival.

Specific Aim 2: Determine the requirement for donor T cell persistence at the tumor site to
promote long-term survival

Previous results demonstrated donor T cell persistence in the brain in association with
long-term survival (117). Here, our goal was to determine the requirement for donor T cell
persistence in maintaining long-term survival. To test this, we utilize anti-CD8 depleting antibody
to deplete CD8+ cells from ACT-treated mice and assess tumor control.
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Figures and figure legends

Figure 2-1 Illustration of an anti-tumor T cell immune response. Tumor-specific T cell responses are initiated by dendritic cells (DC) that present tumor-derived neoantigens to T cells. Activated cytotoxic T lymphocyte (CTL) effectors eliminate tumor cells presenting the neoantigen.
Figure 2-1
Figure 2-2 Anti-tumor T cell responses can be disrupted by multiple resistance mechanisms. 1. Tumor cell clones which lose expression of the neo-antigen avoid detection by tumor-specific CTLs. 2. Tumor cells which downregulate surface expression of MHC class I molecules and other immune targets conceal themselves from immune recognition. 3. Upregulation of anti-apoptotic proteins such as the Bcl-2 family renders tumor cells resistant to immune-induced apoptosis. 4. Tumor cells inhibit CTL effector function by expression inhibitory ligands such as PD-L1 at the cell surface. 5. The tumor microenvironment (TME) is rich in immunosuppressive cytokines which are secreted by regulatory T cells (Ts) and myeloid-derived suppressor cells (MDSC). 6. MDSCs also produce immunosuppressive enzymes which deplete the TME of metabolites important for CTL function.
Figure 2-2
Figure 2-3 Carcinogen-induced tumors develop differently in immune-competent and immune-deficient due to cancer immunoediting. *a*, Tumors grown in immune-competent mice are edited and 100% progress following passage to immune-competent secondary recipient mice. *b*-c, Tumors grown in immune-deficient mice are not edited. Following passage to immune-competent secondary recipient mice, 60% of unedited tumors were rejected (*b*) and 40% grew progressively (*c*). The schematic is adapted from Schreiber et al. *Science*, 2011.
Figure 2-3
Figure 2-4 Schematic of therapeutic protocol for adoptive T cell transfer therapy with tumor-infiltrating lymphocytes (ACT-TIL therapy). 1, Tumor tissue is surgically removed. 2, Surgical tumor specimen is disaggregated and enzymatically digested before culture in media containing IL-2 to promote outgrowth of tumor-infiltrating lymphocytes (TIL). 3, Recovered TIL are rapidly expanded in culture to obtain approximately $10^{11}$ T cells for re-infusion. 4, The day before adoptive T cell transfer (ACT), the patient receives a lymphodepleting host-conditioning regimen of chemotherapy +/- whole-body irradiation. 5, On the day of ACT, expanded T cells are re-infused into the lymphodepleted patient and supplemented with IL-2.
Rapid expansion to $10^{11}$ T cells

Selective expansion of TIL in culture

Re-infusion of T cells into patient followed by IL-2

Surgical resection of tumor tissue

Host-conditioning with lymphodepleting regimen

Tumor cell

Tumor-infiltrating lymphocyte (TIL)

Figure 2-4
Figure 2-5 Lymphodepletion enhances ACT therapy by depleting immunosuppressive cells from the tumor microenvironment (TME). a, Immunosuppressive T\textsubscript{REG} cells and MDSCs inhibit TIL effector function in the TME. b, Lymphodepletion eliminates suppressive cells from the TME and promotes TIL-mediated tumor elimination.
Figure 2-5

a  Tumor microenvironment before host-conditioning

b  Tumor microenvironment following host-conditioning

- Regulatory T cell (T_{REG})
- Myeloid-derived suppressor cell (MDSC)
- Tumor cell
- Tumor-infiltrating lymphocyte (TIL)
Figure 2-6 Lymphodepletion enhances ACT therapy by increasing T cell access to important stimuli. 

**a,** In the lymphoreplete host, non-specific T cells and NK cells consume homeostatic cytokines and may impede access to antigen-presenting cells (APCs). 

**b,** In the lymphopenic host, the availability of homeostatic cytokines and APCs promotes the activation and accumulation of adoptively-transferred tumor-specific T cells.
ACT following host lymphodepletion

ACT without host lymphodepletion

Figure 2-6

- Antigen-presenting cell
- Tumor-specific T cell
- Non-specific T cell
- NK cell
- Homeostatic cytokine
Figure 2-7 Chemotherapy- or irradiation-induced damage following host-conditioning promotes T cell immunity. a, Damaged or dying tumor cells release tumor antigens and cell-derived danger signals. Damage to the intestinal lumen creates microperforations, allowing the translocation of gut-derived microbial danger signals into circulation. b, Danger signals stimulate antigen-presenting cells (APCs) to present tumor antigens and activate T cells.
Figure 2-7

- Damage induced by host-conditioning
- Microbial danger signals
- Tumor-derived danger signals
- Tumor antigen
**Figure 2-8 Dendritic cell (DC) licensing by anti-CD40 therapy.**  
*a*, Resting DC does not express the appropriate costimulatory molecules or cytokines to promote T cell activation.  
*b*, Agonist anti-CD40 engages the CD40 receptor, licensing the DC to promote T cell activation through expression of costimulatory molecules and cytokines.
Figure 2-8
**Figure 2-9 Schematic of anti-CTLA-4 therapy.**

*a,* Engagement of the CTLA-4 inhibitor receptor on activated T cells suppresses activation of anti-tumor immunity. *b,* Anti-CTLA-4 therapy blocks engagement of the CTLA-4 receptor, releasing the activated T cell from inhibition.
Figure 2-9
Figure 2-10 Anti-PD-1 therapy reverses tumor cell resistance to T cell-mediated cytotoxicity. 

a, A tumor-specific T cell identifies and eliminates a PD-L1<sup>-</sup> tumor cell. 
b, A PD-L1<sup>+</sup> tumor cell inhibits T cell-mediated cytotoxicity. 
c, Anti-PD-1 therapy blocks engagement of the PD-1 receptor and the tumor cell is eliminated.
Figure 2-10
Figure 2-11 The T Ag determinants recognized by CD8\(^+\) T cells. 

\(a\), The four CD8\(^+\) T cell determinants of T Ag are listed with their corresponding amino acid location, sequence, and MHC class I restriction. 

\(b\), This illustration depicts the four known CD8\(^+\) T cell determinants of the 708 amino acid T Ag protein.
<table>
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<th>Restriction</th>
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**Figure 2-11**
Chapter 3

Influence of timing and location of irradiation host-conditioning on adoptive T cell immunotherapy

Abstract

Adoptive T cell transfer (ACT) using CD8+ T cells has been successfully used to treat multiple cancer types and can be curative when coupled with lymphodepleting chemotherapy or whole-body irradiation (WBI). Given the immune-privileged nature of the brain, we hypothesized that the local effects of WBI conditioning on the brain were critical in inducing a window of opportunity during which the tumor was sensitive to donor T cell accumulation and regression. To test this hypothesis, we analyzed donor T cell accumulation following ACT in mice bearing autochthonous brain tumors at varying intervals following WBI. We show that WBI conditioning creates a prolonged window of opportunity for ACT therapy, however, maximum protection from recurrence required ACT early after WBI. We also compared ACT therapy in mice conditioned with irradiation localized to the body or to the head. We show that irradiation of the body, but not the head, was sufficient to replicate the therapeutic effects observed using WBI, including complete tumor regression. Local irradiation of the head enhanced donor T cell accumulation in the tumor-draining lymph nodes and provided a modest survival benefit but did not promote long-term tumor control. These observations illustrate the importance of the systemic effects of WBI conditioning in promoting ACT-mediated tumor control, even within an immune-privileged site.

Introduction

Adoptive T cell transfer (ACT) therapy is a promising cancer immunotherapy that can mediate significant tumor regressions and has achieved objective and complete responses in
cancer patients at a rate higher than standard treatments (1). ACT therapy involves the adoptive transfer of high numbers of autologous, tumor-reactive T cells into cancer patients to mediate an anti-tumor immune response. Autologous, tumor-reactive T cells are derived from either tumor-infiltrating lymphocytes, which are isolated \textit{ex vivo} from surgically removed tumor specimens and expanded in culture, or from lymphocytes isolated from peripheral blood and genetically engineered to recognize tumor antigens (2). A landmark development in ACT therapy has been the use of lymphodepleting host-conditioning regimens, which support the survival and efficacy of the transferred T cells when administered to cancer patients prior to T cell reinfusion (3). In clinical trials, host-conditioning regimens originally consisted of chemotherapy, however, the use of sub-lethal whole-body irradiation (WBI) in host-conditioning regimens has been shown in animal models and clinical trials to improve response rates and durability of ACT therapy (4,5).

The mechanisms underlying WBI-enhanced ACT therapy remain incompletely defined. WBI conditioning induces a number of changes in tumor-bearing hosts, both systemically and locally at the tumor site, that have been proposed to contribute to WBI-enhanced ACT therapy. The systemic mechanisms shown to be critical include the lymphodepletion of endogenous immune cell populations that suppress T cell immunity through direct inhibitory mechanisms or through consumption of important T cell cytokines (6,7), as well as the systemic activation of innate immunity by microbial products released by damaged intestinal mucosa (8). Locally, irradiation has been shown to promote the activation of innate and adaptive immune cells in the tumor microenvironment (TME) and the tumor-draining lymph node, resulting in enhanced presentation of tumor-derived antigens, chemokine production, immune cell infiltration, and potent effector function (9-11). Notably, immune infiltration and tumor control following local irradiation was significantly reduced in mice that lack expression of the type I IFNαβ-receptor on host cells, but not tumor cells (11). This demonstrates a critical role for type I IFN in activating the host response to local irradiation. Local type I IFN levels were significantly increased at two
days but not four days post-irradiation, suggesting this local effect is short-lived.

Our group has previously shown that WBI conditioning dramatically improves the efficacy of ACT therapy in the treatment of autochthonous murine brain tumors (12,13). Line SV11 mice develop autochthonous brain tumors due to high-level transgenic expression of the simian virus 40 (SV40) large T antigen oncoprotein under control of the viral promoter in the choroid plexus of the brain (14). Choroid plexus tumors are microscopically detectable beginning at approximately 35 days of age and progress to become lethal at approximately 104 days of age (15). T antigen is also expressed at low-levels in the kidney but does not result in tumor formation there (14). Due to low-level transgene expression in the thymus (unpublished observations), SV11 mice are immunologically tolerant to T antigen and unable to mount an endogenous T antigen-specific CD8+ T cell response. Tolerance can be overcome by ACT of TCR-transgenic T cells that express TCRs specific for the known T antigen determinants, including the immunodominant site IV determinant (residues 404-411). Treatment of 80 day-old SV11 mice bearing established choroid plexus tumors with ACT using naïve TCR-transgenic CD8+ T cells targeting the site IV determinant (TCR-IV T cells) can mediate modest tumor control, however, the addition of host-conditioning with 4 Gy WBI one day prior to ACT results in rapid accumulation of donor T cells in the lymphoid organs and brain, regression of established tumors, and long-term protection from tumor recurrence in association with donor T cell persistence (13).

Here, we sought to understand the underlying mechanisms of WBI conditioning that promote ACT therapy of established brain tumors by dissecting the local and systemic effects of WBI. We hypothesized that the local effects of WBI conditioning on the brain were critical in inducing a brief window of opportunity for donor T cell accumulation in the brain and tumor regression. Previous studies have demonstrated a requirement for local irradiation in promoting T cell-mediated brain tumor therapy (16,17), however the impact of the timing of ACT and irradiation conditioning has not been investigated. We also show that irradiated brain tumors
remain susceptible to ACT-mediated regression even when ACT is delayed following WBI conditioning, however, long-term therapeutic benefit was maximized when ACT was administered early after WBI. Furthermore, we show that irradiation conditioning promotes ACT therapy through both local and systemic effects, but that only the systemic effects are required to promote ACT-mediated tumor regression and protection from tumor recurrence.

**Materials and Methods**

**Mice**

SV11 mice (18) (C57BL/6-Tg(TAg)11Bri) were maintained as previously described (19) and used at 75-85 days of age in all experiments. B6.Cg-Tg(TcraY4,TcrbY4)2025Tdsc, or TCR-IV, mice express a T cell receptor (TCR) αβ pair specific for the H2-K\(^b\) restricted site IV epitope (13). All mice were maintained in specific pathogen-free conditions at the Milton S. Hershey Medical Center animal facility. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Penn State Hershey College of Medicine.

**Host conditioning and adoptive T cell transfer**

WBI-conditioned mice were administered 4 Gy WBI on day -1 using a \(^{60}\)Co Gammacell irradiator (Nordion International). For local irradiation experiments, all mice were anesthetized with 150 µL ketamine/xylanine (70 mg/kg and 10 mg/kg, respectively in sterile PBS) i.p. and then administered either no radiation or 4 Gy x-ray radiation using an X-RAD 320ix biological x-ray irradiator (Precision X-Ray Inc.). Anesthetized mice were placed in a prone position and aligned with the edge of the collimator beam at the base of the ears. Body irradiation (BI)-conditioned
mice received 4 Gy x-ray radiation below this line, and head irradiation (HI)-conditioned mice received 4 Gy radiation above this line. Total cell counts in cervical lymph nodes at the time of tissue harvest confirmed that the cervical lymph nodes were within the irradiation field during BI, but not HI conditioning. Whole-cell populations were recovered from spleens and axillary, brachial, superficial cervical, mesenteric, inguinal, and lumbar lymph nodes (20) of TCR-IV donor mice. CD8+ cells were enriched by autoMACS magnetic sorting using the manufacturer’s recommendations (Miltenyi Biotec), resulting in approximately 90% pure CD8+ TCR-IV+ T cells. 1 x 10^6 naïve TCR-IV T cells were administered intravenously in 200 µL PBS on day 0 of the experiments. For CFSE labeling, sorted donor T cells were resuspended (10^7 cells/mL) in PBS containing 0.1% BSA. CFSE was added to a final concentration of 5 µM and vortexed immediately. Following 10 minute incubation at 37°C, cells were washed three times before ACT.

**Lymphocyte isolation and flow cytometric analysis**

On the day of analysis, spleens, superficial cervical lymph nodes (cLN), and brains were removed from CO2-euthanized mice following exsanguination. Lymphocytes from spleens and cLN were obtained as previously described (19). Briefly, spleens and cLNs were mechanically disrupted by pressing tissue through metal screens to obtain single-cell suspensions in approximately 7 mL cold RPMI-1640 (henceforth called RPMI) with GlutaMAX™ (Gibco) supplemented with 2% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 10 mM HEPES, and 25 µg/mL pyruvic acid. Red blood cells (RBCs) were eliminated by 5 minute incubation in warm tris ammonium chloride. Following RBC-depletion, white cells were washed once in cold RPMI, then resuspended in 1-5 mL cold RPMI for live cell counting by trypan blue exclusion. Prior to removing spleen and cLN cell aliquots for
antibody staining, samples were left on ice for 5 minutes to allow large debris to settle. Brain lymphocytes were obtained as previously described (12). Briefly, brains were minced with a razorblade and mixed by pipetting in cold RPMI. Dispersed cells were placed on ice and large debris allowed to settle twice for 5 minutes, after which the supernatant containing cells was collected. The single-cell suspension was RBC-depleted and washed before centrifugation (500 × g for 20 minutes) on a density gradient (Percoll, 70%:32%). Cells at the interface were removed with a Pasteur pipet, washed, and live cells counted by trypan blue exclusion. Fluorochrome-labeled antibodies were obtained from eBioscience (CD8α clone 53-6.7, CD44 clone IM7, killer-cell lectin like receptor G1 (KLRG1) clone 2F1), BD Biosciences (CD45.2 clone 104, CD127 clone SB/199), BioLegend (CD62L clone MEL-14), and Tonbo Biosciences (CD8α clone 53-6.7). Site IV/Kb and gB/Kb tetramers were prepared and used to stain site IV- or gB-specific T cells as previously described (13,21). Briefly, cells were plated in 200 µL at a density no greater than 1x10⁷ cells/mL in RPMI in round-bottom, tissue culture-treated 96-well plates. Plated cells were washed 2 x 150 µL cold FACS buffer (PBS + 2% FBS + 0.125% sodium azide). After each wash, cells were centrifuged (1400 rpm, 2 minutes, 4°C), and supernatant was removed by swiftly overturning the plate and blotting facedown on a clean paper towel. Cells were blocked in 50 µL cold FACS buffer containing Fc at a 1:100 dilution (10 minutes, room temperature), then washed 1 x 150 µL cold FACS buffer before antibody/tetramer staining. Surface staining with antibodies and tetramers was performed simultaneously in 50 µL cold FACS buffer (15 minutes, room temperature, protected from light). Antibodies and tetramers were used at a 1:100 dilution. Following staining, cells were washed 3 x 150 µL FACS buffer and resuspended in 200-300 µL cold FACS buffer for flow cytometry. Data were acquired using an LSR II SORP, LSRFortessa or FACSCanto II (BD Biosciences) flow cytometer in the Penn State Hershey Flow Cytometry Core Facility. Data analyses were performed with FlowJo software (TreeStar Inc.).
**Intracellular cytokine staining**

Isolated lymphocytes were incubated with T Ag site IV 404-411 peptide variant C411L (VVYDFLKL) or control herpes simplex glycoprotein B 498-505 peptide (SSIEFARL) for 5-6 hours at 37°C in the presence of Brefeldin A (Sigma). Following incubation, cells were stained for surface markers as described above, then fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen). For cytokine staining, cells were subsequently incubated with fluorochrome-conjugated antibodies for 15 min at room temperature, washed 3 times, and analyzed by flow cytometry. Fluorochrome-conjugated antibodies were obtained from eBioscience (interferon (IFN)γ clone XMG1.2).

**Survival analysis**

Median lifespan was determined by monitoring mice for the development hydrocephalus and neurological signs indicative of advanced tumor development such as lethargy and ataxia (19). Symptomatic mice were euthanized and Kaplan-Meyer survival curves were created using GraphPad Prism software (GraphPad Prism Software, Inc.).

**Histology and immunohistochemistry**

Following sacrifice, mice were perfused with PBS followed by 10% neutral buffered formalin (NBF). Brains from perfused mice were removed and stored overnight in NBF and then transferred to 70% ethanol. Fixed brains were paraffin embedded and representative coronal sections were collected throughout the brain. Brain sections were hematoxylin and eosin (H&E) stained and the maximum tumor diameter was determined for each mouse by light microscopy using a Nikon CFW10X eyepiece. Images were captured using a Nikon Microphot-FXA.
microscope with a 10x objective fitted with a Sony DKC-ST5 Digital Photo Camera and Sony DKC-ST5 imaging software version 1.10.

**Statistics**

All statistical tests were performed using GraphPad Prism software. Unpaired Student’s $t$-test was used to determine significance unless otherwise noted. A $p$-value less than 0.05 was considered statistically significant and is indicated using *, **, ***, **** ($p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively).

**Results**

*Delay of ACT by up to seven days post-WBI does not compromise acute tumor regression*

The previous finding that donor T cells accumulate rapidly in the brains of WBI-conditioned but not unirradiated mice (13) led us to hypothesize that local, short-lived effects of irradiation were required for rapid donor T cell accumulation and regression of established brain tumors. We first asked whether administering delayed ACT after WBI conditioning would compromise ACT-mediated acute tumor regression. To test this, groups of 80 day-old SV11 mice received WBI conditioning on day -10, -7, -4, or -1 followed by TCR-IV ACT on day 0 (Fig. 3-1a). Ten days later, mice were sacrificed for histological analysis of formalin-fixed brain sections, as well as flow cytometry analysis. We observed tumors partially or completely replaced by stromal condensation, indicative of tumor regression, in mice that received ACT delayed by as much as ten days (Fig. 3-1b). We calculated the area of residual tumor lesions by measuring cross-sections of the largest lesion in each brain and plotted these values (Fig. 3-1c). In agreement
with previous results, all mice in the WBI(day -1)+ACT group were tumor-free. While there was no statistical difference in tumor area amongst the treatment groups, there was a trend toward a higher rate of incomplete tumor regression in groups with lengthier delay between WBI and ACT, with over 50% of mice in the WBI(day -10)+ACT group having residual tumor lesions on day +10 (Fig. 3-1d). Incomplete tumor regression may indicate impaired donor T cell accumulation or effector function at the tumor site as a result of delayed ACT. We concluded that seven days was the maximum duration of delay between WBI and ACT that did not compromise acute tumor regression in SV11 mice.

**Delay of ACT following WBI does not compromise donor T cell accumulation on day +10**

Using flow cytometry, we determined the magnitude of donor T cell accumulation by frequency and by total number in the spleens of the mice in Figure 3-1. For each treatment group, additional mice were included in which both spleen and brain were analyzed by flow cytometry. By percentage, there was no statistical difference in donor T cell accumulation in the spleen between groups receiving delayed ACT and the WBI(day -1)+ACT group (Fig. 3-2a-b). Additionally, we did not observe any reduction in total donor T cell accumulation by number in groups receiving delayed ACT (Fig. 3-2c). Unexpectedly, we observed a modest but statistically significant increase in total donor T cell accumulation by number in the WBI(day -10)+ACT group compared to the WBI(day -1)+ACT group (Fig. 3-2c); this finding reflects an increase in total spleen counts in this group (unpublished observation). In the brains, donor T cells were readily detectable by tetramer-IV staining in all mice analyzed and had accumulated to a similar frequency (Fig. 3-2d). To determine whether delay in ACT had any impact on the activation and differentiation of donor T cells following adoptive transfer, we also stained spleen and brain flow cytometry samples for expression of the CD44 adhesion molecule, up-regulated following antigen
encounter, and the L-selectin (CD62L) lymph node homing receptor, down-regulated by effector T cells. The majority of donor T cells displayed a similar CD44^{hi}CD62L^{lo} activated effector phenotype in all groups in both the spleen and brain (Fig. 3-2e), demonstrating no observable differences among treatment groups in donor T cell differentiation on day +10 post-ACT. We concluded that delay in ACT following WBI conditioning does not compromise donor T cell activation, accumulation, or differentiation in the spleen or the brain on day +10.

**Delay of ACT does not compromise tumor elimination and donor T cell persistence on day +30**

Because tumors did not fully regress in some mice by day +10, we sought to confirm that mice treated with WBI(day -7)+ACT and WBI(day -1)+ACT therapy achieved similar levels of tumor elimination and donor T cell persistence at a later time point. Groups of mice received either WBI(day -7)+ACT or WBI(day -1)+ACT therapy and were sacrificed on day +30 to assess tumor burden and donor T cell persistence in the lymphoid organs. All mice appeared healthy at the time of sacrifice, and upon examination of H&E stained brain sections all mice were found to be tumor-free (Fig. 3-3a). Flow cytometry analysis of spleens and cLNss revealed low but readily detectable tetramer-IV^{+} donor T cells in mice from both groups (Fig. 3-3b). Tetramer-IV^{+} donor T cells were not detectable in one mouse from the WBI(day -7)+ACT group, however we did observe stromal condensation in the brain (Fig. 3-3a, left), demonstrating successful ACT-mediated tumor regression. Total accumulation of donor T cells by number and percentage were approximately equivalent in both groups (Fig. 3-3c). Thus, we observed no difference in tumor burden or donor T cell persistence between mice treated with WBI(day -7)+ACT and WBI(day -1)+ACT on day +30. These results indicate that complete tumor regression and donor T cell persistence is achieved in mice receiving either WBI(day -7)+ACT or WBI(day -1)+ACT therapy.
**ACT early after WBI conditioning is required to maximize therapeutic benefit**

We originally hypothesized that the effects of irradiation conditioning on the tumor microenvironment created a limited window of time following WBI conditioning during which the tumor was susceptible to ACT therapy. The finding that tumors were completely regressed in WBI(day -7)+ACT and WBI(day -1)+ACT treated mice by day +30 led us to hypothesize that both therapies would promote long-term survival and protection from tumor recurrence. To determine whether delay in ACT following WBI conditioning would compromise the long-term therapeutic efficacy of WBI+ACT therapy, groups of SV11 mice were treated at 80 days of age with either WBI(day -7)+ACT or WBI(day -1)+ACT therapy. Additional control groups remained untreated or received only WBI. Mice were monitored for signs of tumor outgrowth such as hydrocephalus, ataxia, or weight loss and euthanized when appropriate. WBI treatment alone provided no survival benefit compared to untreated mice (Fig. 3-4a-b), with median lifespans reaching 115 days and 111 days, respectively. Meanwhile, survival was significantly extended in both the WBI(day -7)+ACT and WBI(day -1)+ACT treatment groups compared to untreated mice. Interestingly, overall survival was significantly extended in the WBI(day -1)+ACT group compared to the WBI(day -7)+ACT group (Fig. 3-4a-b), with median lifespans reaching 306 days and 212 days, respectively. We performed flow cytometry on the brains of two mice in the WBI(day -7)+ACT group at age 212 days. Both mice exhibited signs of tumor recurrence and had grossly visible tumors upon necropsy. Persistent donor T cells were detected in the brains of both mice by tetramer-IV staining at this time point (Fig. 3-4c). Additionally, a fraction of CD8+ T cells were observed to produce IFNγ following incubation with site IV peptide (Fig. 3-4c), suggesting a subset of donor T cells retained the ability to respond to tumor antigen recognition. Collectively, these results demonstrate that ACT can be administered at least seven days post-WBI conditioning and still promote significantly prolonged survival in association with
the persistence of functional donor T cells. Administration of ACT early after WBI conditioning, however, is required to achieve maximum therapeutic benefit.

We next sought to explain the difference in long-term therapeutic benefit between WBI(day -7)+ACT and WBI(day -1)+ACT treatment groups despite only minor observable differences in tumor regression or donor T cell accumulation on day +10 or +30. A previous study from our group demonstrated significant differences in early donor T cell accumulation and long-term survival for SV11 mice receiving vaccination on day 0 versus day +7 post-ACT with donor T cells targeting the site V determinant (22). This finding suggested that the timing of ACT therapeutic protocols influences early donor T cell accumulation and long-term survival. Therefore, we hypothesized that delay of ACT following WBI conditioning may compromise donor T cell accumulation and differentiation early after ACT.

**Initial proliferation of donor T cells is not impacted by WBI conditioning**

One possible explanation for the difference in therapeutic benefit is differences in initial *in vivo* proliferation of donor T cells. We hypothesized that a reduction in initial proliferation may impair subsequent early accumulation at the tumor site. To determine whether initial proliferation of TCR-IV donor T cells was impaired when ACT was delayed following WBI conditioning, we analyzed donor T cell proliferation on day +2 post-ACT. Prior to ACT, donor T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE), a fluorescent tracking dye which is diluted each time the cell divides, allowing assessment of donor T cell proliferation. Groups of SV11 mice received WBI(day -7)+ACT or WBI(day -1)+ACT therapy with CFSE-labeled TCR-IV donor T cells. A third group of SV11 mice received ACT without WBI conditioning, and a control group of T antigen transgene-negative littermates (B6 mice) that received WBI(day -
1)+ACT treatment was also included. On day +2, mice were sacrificed to assess CFSE-dilution in splenic donor T cells by flow cytometry. Few donor T cells were detectable in the cLN at this early time point (data not shown). In control B6 mice, the majority of donor T cells in the spleen remained undivided on day +2 (Fig. 3-5a-b). In contrast, a majority of donor T cells had divided in both the WBI(day -7)+ACT and WBI(day -1)+ACT groups (Fig. 3-5a-b). A similar fraction of donor T cells in unirradiated SV11 mice had also divided (Fig. 3-5a-b), suggesting WBI conditioning does not enhance initial proliferation of donor T cells. These data indicate that delay of ACT following WBI conditioning does not impair initial donor T cell proliferation. Furthermore, the reduction in long-term therapeutic benefit in mice receiving delayed ACT is not associated with impaired initial donor T cell proliferation.

**Early donor T cell accumulation is impaired following delayed ACT**

To account for the reduction in long-term therapeutic benefit in mice receiving delayed ACT, we sought to determine whether early donor T cell accumulation in the lymphoid organs and the brain was impaired in these mice, as our group has previously demonstrated that early donor T cell accumulation in the brain is associated with acute tumor regression (23). To determine whether early accumulation was impaired, groups of SV11 mice received either WBI(day -7)+ACT or WBI(day -1)+ACT therapy and were analyzed on day +5 by flow cytometry to determine the magnitude of donor T cell accumulation in spleen and brain. On day +5, donor T cells were detectable by tetramer-IV staining in the spleens of all mice and displayed a similar CD44<sup>hi</sup>CD62L<sup>lo</sup> activated effector phenotype (Fig. 3-6a), indicating that delay in ACT did not impact the differentiation of donor T cells. By percentage, donor T cell accumulation in the spleens of the WBI(day -7)+ACT group was significantly lower compared to the WBI(day -1)+ACT group (Fig. 3-6b), although this difference was not borne out when the total number of
donor T cells was calculated (Fig. 3-6c). Total cell counts in spleens from the WBI(day -1)+ACT group were significantly reduced compared to the WBI(day -7)+ACT group, demonstrating moderate recovery from lymphodepletion in WBI(day -7)+ACT treated mice (Fig. 3-6d).

Trends in donor T cell accumulation in the brains mirrored those in the spleens on day +5, with a significant enhancement in donor T cell accumulation in the WBI(day -1)+ACT group by percentage, but not by total donor T cell number (Fig. 3-7a-c). Importantly, donor T cells were detectable in the brains of all mice, demonstrating that delay in ACT did not wholly impair initial accumulation of donor T cells in the brain when ACT was delayed. There was also no significant difference in the total brain cell counts from Percoll-enriched brain cell fractions (Fig. 3-7d), suggesting the low percentage of donor T cells accumulating in the brains of mice in the WBI(day -7)+ACT group was not due to an influx of other immune cells into the brain. The majority of donor T cells in both groups expressed a similar CD44 hi CD62L lo activated effector phenotype, suggesting that delay in ACT did not significantly impact the differentiation of donor T cells (Fig. 3-7a). The significant reduction in donor T cell accumulation by percentage and the trend toward reduced total donor T cell numbers suggest early donor T cell accumulation in the spleen and brain is impaired when ACT is delayed following WBI conditioning, although this impairment only reached statistical significance by percentage.

**Delay of ACT following WBI conditioning reduces antigen-independent homeostatic proliferation of donor T cells**

The finding that the early accumulation of donor T cells was impaired in mice treated with WBI(day -7)+ACT compared to WBI(day -1)+ACT despite similar initial donor T cell proliferation rates suggested the survival of donor T cells in the latter group was enhanced. Given the higher spleen counts in the WBI(day -7)+ACT treated mice, we hypothesized that donor T
cells proliferating in these mice encounter increased competition for survival cytokines with endogenous lymphoid cells, resulting in a lower rate of homeostatic proliferation and impaired donor T cell accumulation.

Therefore, we assessed the contribution of antigen-independent homeostatic proliferation on donor T cell proliferation following ACT into WBI-conditioned mice. To test this, groups of SV11 mice received WBI(day -7)+ACT or WBI(day -1)+ACT treatment with CFSE-labeled control TCR-transgenic T cells (TCR-gB) specific for herpes simplex glycoprotein B epitope 498-505. On day +5, the lymphoid organs were analyzed by flow cytometry using H-2K\textsuperscript{b}/HSV glycoprotein B tetramer-staining (tetramer-gB) to identify TCR-gB donor T cells (Fig. 3-8a). Because the CFSE signal is diluted each time the cell divides, reduction in the mean fluorescence intensity (MFI) of CFSE labeling in tetramer-gB\textsuperscript{+} TCR-gB donor T cells was used as a read-out for donor T cell proliferation. In both the spleen and cLN, MFI levels of donor TCR-gB T cells from mice in the WBI(day -1)+ACT group were significantly lower compared to WBI(day -7)+ACT group (Fig. 3-8a,c), indicating donor T cells had undergone additional rounds of division in the former group. Donor T cells in both groups expressed a CD44\textsuperscript{hi}CD62L\textsuperscript{hi} phenotype consistent with naïve T cells undergoing homeostatic proliferation (Fig. 3-8b). These results indicate that WBI conditioning promotes the antigen-independent homeostatic proliferation of donor T cells and that this effect is more robust when ACT occurs early after irradiation conditioning.

Collectively, these findings imply that the systemic effects of WBI conditioning promote enhanced donor T cell accumulation and maximum therapeutic benefit when ACT is administered early after WBI conditioning, however they do not rule out the possibility that local effects of WBI on the TME are required to achieve tumor regression and protection from recurrence. Thus, we undertook a series of experiments to define the contributions of local versus systemic irradiation conditioning in ACT therapy.
**Irradiation conditioning focused to the body is sufficient to promote rapid accumulation of donor T cells in the lymphoid organs and at the tumor site**

We hypothesized that local effects of WBI on the brain and TME may contribute to the rapid accumulation of donor T cells on day +5. Thus, we asked whether irradiation conditioning focused to the body (body irradiation, BI) or to the head (head irradiation, HI) was sufficient to reproduce the early day +5 accumulation of TCR-IV donor T cells in the lymphoid organs and the brain previously observed using WBI conditioning (13). Groups of SV11 mice received either HI, BI, or no conditioning one day prior to ACT with naïve TCR-IV T cells (Fig. 3-9a). Mice were sacrificed on day +5 post-ACT to assess donor T cell accumulation in the spleen, cLN, and the brain. By day +5, BI conditioning induced significant lymphodepletion in the spleen and cLN, while HI conditioning did not significantly impact total cell counts (Fig. 3-9b-c). In mice receiving either HI or BI, the frequency of tetramer-IV+ donor T cells in the lymphoid organs was enriched compared to mice receiving no irradiation (Fig. 3-9d-g); this trend was particularly pronounced in BI-conditioned mice. In the brain, however, only mice receiving BI conditioning showed enrichment for tetramer-IV+ donor T cells (Fig. 3-9d,g). We calculated the total numbers of donor T cells in each tissue to determine whether this enrichment by percentage correlated with enhanced accumulation by number. Donor T cells accumulated to higher levels on day +5 in the lymphoid organs and brains of BI-conditioned mice compared to unconditioned mice (Fig. 3-9h-j). HI conditioning enhanced donor T cell accumulation only in the cLN (Fig. 3-9h-j). These results demonstrate that BI conditioning is sufficient to promote enhanced early accumulation of donor T cells in the lymphoid organs and brains of SV11 mice, similar to previous results with WBI conditioning (13). Conversely, HI conditioning, which includes irradiation of the tumor, enhances early donor T cell accumulation in the tumor-draining cLN but not the spleen or the brain.

Donor T cells accumulating in the lymphoid organs and brains of mice in all groups
expressed phenotypic surface marker patterns consistent with T cell activation and differentiation (Fig. 3-10a-c). Donor T cells uniformly expressed the CD44 adhesion molecule, a marker of T cell activation. Additionally, a substantial fraction of donor T cells had down-regulated the L-selectin lymph node homing receptor (CD62L) and the IL-7 receptor \( \alpha \) chain (CD127), commonly expressed by naïve T cells and down-regulated during effector differentiation. Few donor T cells up-regulated expression of killer cell lectin-like receptor G1 (KLRG1), a marker of terminally differentiated effector T cells. Thus, donor T cells displaying phenotypic markers of activation and early differentiation (CD44\(^{hi}\), CD62L\(^{lo}\), CD127\(^{lo}\), KLRG1\(^{-}\)) were present in all mice by day +5 post-ACT.

To quantify the extent of effector-differentiation, we calculated the frequency of CD44\(^{hi}\)CD62L\(^{lo}\) effector T cells within the donor T cell population. The donor T cell population was significantly enriched for effector T cells in the lymphoid organs and brains of BI-conditioned mice compared to unconditioned mice (Fig. 3-10d-f). In contrast, HI conditioning resulted in effector T cell-enrichment in the cLN, but not the spleen or brain (Fig. 3-10d-f).

Collectively, these results indicate that donor T cells undergo early priming and differentiation in all groups, regardless of irradiation conditioning. However, BI conditioning accelerated effector-differentiation of donor T cells in spleen, cLN, and brain, while local irradiation of the tumor site in HI-conditioned mice only promoted enhanced differentiation locally in the cLN.

**Irradiation conditioning focused to the body is sufficient to promote enhanced donor T cell accumulation and ACT-mediated tumor regression independently of irradiation of the tumor**

We next asked whether HI or BI conditioning was sufficient to promote ACT-mediated acute tumor regression as previously observed using WBI conditioning (13). We also sought to determine the impact of these conditioning regimens on donor T cell accumulation at this later
time point. Groups of unconditioned, HI-conditioned, or BI-conditioned mice received TCR-IV ACT and were sacrificed on day +10 post-ACT. Donor T cells in spleens and cLN were assessed by flow cytometry, and formalin-fixed brain sections were examined following H&E staining. In the spleen, donor T cell accumulation was enhanced in BI-conditioned mice, but not in HI-conditioned mice compared to unconditioned mice (Fig. 3-11a). Similar to day +5 (Fig. 3-9g), donor T cell accumulation was significantly higher in cLN of BI-conditioned mice (Fig. 3-11b). In contrast, donor T cell accumulation in cLN on day +10 was not enhanced in HI-conditioned mice (Fig. 3-11b), indicating the early enhancement observed on day +5 was short-lived (Fig. 3-9g).

The expression of surface phenotypic markers followed similar trends as on day +5, with the majority of donor T cells in all three groups displaying a CD44hiCD62LloCD127lo phenotype in the spleen and cLN (Fig. 3-11c-d), and all three groups showed similar frequencies of effector-differentiated donor T cells with modest enrichment in the cLN of BI-conditioned mice (Fig. 3-11e-f). Interestingly, a substantial fraction of donor T cells in BI-conditioned mice expressed KLRG1, suggesting these cells had undergone extensive differentiation (Fig. 3-11c-d).

To determine whether localized irradiation conditioning was sufficient to promote ACT-mediated acute tumor regression by day +10, we examined H&E stained brain sections for evidence of tumor regression (Fig. 3-12a). Indeed, we observed stromal condensation, indicative of tumor regression, in all mice that received BI conditioning (Fig. 3-12a-b). In contrast, there was no evidence of tumor regression in mice that received HI conditioning, similar to unconditioned mice (Fig. 3-12a-b). To quantify this effect, the largest cross-sectional area from each tumor was plotted (Fig. 3-12b), illustrating the therapeutic effect in the BI+ACT group. These results demonstrate that irradiation of the tumor site is not required for ACT-mediated tumor regression in SV11 mice. Additionally, local irradiation of the tumor site had minimal impact on ACT-mediated acute tumor regression.
Irradiation conditioning focused to the body promotes extended ACT-mediated survival in association with donor T cell persistence in the lymphoid organs and the brain

Our group has previously demonstrated extended survival in SV11 mice treated with WBI conditioning plus TCR-IV ACT with a median survival >180 days (13). To ask whether BI or HI conditioning plus TCR-IV ACT was sufficient to promote extended survival in SV11 mice, we treated groups of mice as described in Figure 3-12 and monitored survival. Control groups of untreated mice and mice receiving only irradiation or ACT were included. BI and HI conditioning alone had no significant impact on survival compared to untreated animals (Fig. 3-13a-b). A modest increase in survival was observed in unconditioned mice that received TCR-IV ACT alone, demonstrating irradiation conditioning is not absolutely required to promote modest ACT-mediated tumor control (Fig. 3-13a-b). BI conditioning in combination with TCR-IV ACT was sufficient to promote extended survival in SV11 mice as observed previously with WBI conditioning (Fig. 3-13a-b and (13)). In contrast, all mice that received HI conditioning plus TCR-IV ACT eventually succumbed to tumor outgrowth, despite prolonged survival compared to HI or ACT alone (Fig. 3-13a-b). These results demonstrate that irradiation conditioning prior to ACT does not require irradiation of the tumor site to promote extended survival. Localized irradiation of the tumor does, however, provide modest enhancement of ACT-mediated tumor control.

Previously, our group demonstrated long-term persistence of donor T cells in the lymphoid organs and the brain following WBI conditioning plus TCR-IV ACT in association with extended survival and protection from tumor recurrence in SV11 mice (13). To ask whether BI conditioning was sufficient to promote the establishment of persisting donor T cells, we performed flow cytometry on the spleen, cLN, and brain samples of all surviving mice in the BI+ACT group on day +155 when the survival experiment was terminated. At the time of sacrifice, all mice appeared healthy and without signs of tumor recurrence. Donor T cells were
detected by tetramer-IV staining in the lymphoid organs and brains of all mice (Fig. 3-13c), indicating irradiation of the tumor site is neither required nor sufficient to promote the establishment of persisting donor T cells in the lymphoid organs and the brain. Conversely, BI conditioning was sufficient to establish persistent donor T cells, similar to WBI conditioning.

**Discussion**

Our findings indicate that WBI conditioning can promote effective ACT therapy of established brain tumors even when ACT is delayed following WBI conditioning or when the tumor is not irradiated directly. Irradiation-induced systemic lymphodepletion was required to promote ACT-mediated tumor regression and therapeutic efficacy was optimized when ACT was administered early after WBI conditioning. These findings are in agreement with other studies in animal models and cancer patients showing an association between the therapeutic efficacy of WBI+ACT therapy and the degree of host lymphodepletion (4,5). These findings uniquely demonstrate that tumors located in an immune-privileged site such as the brain are susceptible to robust ACT therapy even when host-conditioning regimens do not modulate the TME.

To our knowledge, the influence of the timing of ACT following WBI conditioning on therapeutic outcome has not been investigated in the setting of ACT therapy of established tumors. Using the SV11 mouse brain tumor model, our group previously demonstrated that a standard therapeutic protocol of sub-lethal WBI conditioning one day prior to ACT with naïve TCR-IV T cells results in a rapid, high-level donor T cell accumulation in the brain, tumor regression, and protection from tumor recurrence (13). Here, we originally hypothesized that the local effects of WBI conditioning on the brain were critical in inducing a window of opportunity during which the tumor was sensitive to ACT-mediated regression, and that the therapeutic efficacy of WBI+ACT therapy would diminish when ACT was delayed following WBI as these
local effects abated. We found that a modified WBI+ACT protocol in which ACT is delayed following WBI conditioning is still therapeutically effective and did not compromise complete tumor regression, donor T cell persistence, or prolonged survival. These findings demonstrate flexibility in the WBI+ACT therapeutic protocol.

Given the observation of complete tumor regressions, we were surprised to find that tumors recurred earlier in mice treated with the modified WBI+ACT protocol, in which ACT was delayed seven days post-WBI. Importantly, tumors ultimately recurred in mice treated with either the standard or modified WBI+ACT protocols, indicating neither therapeutic protocol can prevent recurrence indefinitely. Therefore, the mechanisms leading to recurrence may be similar in both treatment groups but proceeding at different rates.

Tumor recurrence following ACT therapy has been associated with immune evasion via loss of target antigen (24), however, antigen loss is highly unlikely in SV11 mice, as we have never observed loss of T antigen expression despite our extensive experience with this model, likely due to multiple integrations of the T antigen transgene. Tumor immune evasion of T cell-mediated immunity has also been associated with acquired defects in the anti-tumor effector T cell response, such as a reduction in the magnitude of T cell-infiltration (25) or the induction of effector T cell anergy (26). T cell anergy in the TME has been associated with the accumulation of CD4⁺ T_{Reg} cells (27) and the expression of T cell inhibitory receptors and their corresponding ligands, such as PD-1/PD-L1 (28,29). In the B16 mouse melanoma model, tumor recurrence following ACT-mediated regression with CD4⁺ donor T cells was mediated by both T_{Reg} cells and PD-1 receptor expression (30). While the mechanisms of tumor recurrence in SV11 mice treated with WBI+ACT therapy were not formally investigated here, loss of immunological control due to a diminishing number of tumor-infiltrating effector T cells seems likely, as SV11 mice are immunologically tolerant to T antigen and are unable to generate T antigen-specific cells de novo. It is important to note that persistent, functional TCR-IV donor T cells were detected in
progressing tumors during recurrence in mice treated with the modified WBI+ACT protocol, suggesting T cell anergy may also play a role in tumor recurrence.

Why, then, do tumors recur earlier in mice treated with the modified ACT protocol? The specific immune cell populations that mediate long-term protection from tumor recurrence in the SV11 model have not been defined, however, the accumulation and persistence of donor T cells in the brain suggests these cells play a role in establishing this protection. An association between the establishment of persisting tumor-reactive T cells at the tumor site and achieving durable regressions has been demonstrated in other models of ACT therapy (31). Upon ACT, TCR-IV donor T cells were introduced into two distinct host environments defined by immune cell populations that have survived or recovered from lymphopenia induced by WBI either one or seven days prior. We reason that the establishment of immunological protection from tumor recurrence, whether directly or indirectly mediated by donor T cells, is influenced by critical mechanisms affected by the host environment during early donor T cell accumulation. In a lymphopenic host, T cell proliferation is driven by antigen-dependent interactions with APCs and antigen-independent homeostatic peripheral expansion (HPE) mediated by cytokines (32). Following ACT into WBI-conditioned mice, TCR-IV donor T cells undergo initial proliferation at a similar rate regardless of the WBI+ACT protocol employed, suggesting the mechanisms promoting antigen-dependent proliferation are not likely the determinants of the range of protection from recurrence. Antigen-independent HPE of antigen-experienced T cells is mediated by homeostatic cytokines, such as IL-15, which promotes the differentiation and survival of long-lived memory T cells (33). Homeostatic cytokine availability increases following lymphodepletion and is inversely correlated with lymphocyte recovery (34,35); this availability has been shown to support the proliferation of adoptively-transferred T cells in vivo (7). The kinetics of homeostatic cytokine availability following lymphodepletion generally increase for a period immediately following conditioning and then return to baseline during lymphocyte
recovery (35,36). Therefore, donor T cells transferred earlier after WBI enter a host environment in which the surge in homeostatic cytokine production supports the antigen-independent proliferation of donor T cells. T cell expansion in the presence of homeostatic cytokines enhances long-term survival and efficacy of memory T cells (33). Thus, ACT early after WBI may promote not only the early accumulation of donor T cells but also their potential to mediate long-term protection from tumor recurrence due to increased responsiveness to IL-15. Future investigation will be required to define the characteristics of the lymphodepleted host environment that optimize donor T cell survival and long-term therapeutic efficacy.

The finding that long-term survival was maximized when ACT was administered early after WBI in association with enhanced early donor T cell accumulation suggests systemic lymphodepletion secondary to WBI conditioning is an important determinant of therapeutic success. However, these findings alone do not preclude the possibility that WBI conditioning also induces local changes to the TME important for ACT therapy.

Previous studies from other groups investigating the underlying mechanisms of irradiation-conditioning and ACT therapy in mouse models have provided conflicting results with regard to the importance of local irradiation of the TME. Using a mouse model of melanoma, Gattinoni et al. showed that shielding the tumor during 5 Gy WBI conditioning had no impact on ACT-mediated tumor control, but that a key mechanism underlying WBI-enhanced ACT therapy was systemic lymphodepletion (7). A separate study by Plautz et al. showed that 5 Gy local irradiation of the tumor site was of primary importance in promoting successful ACT therapy in a mouse brain tumor model (16). Using irradiation focused to the head or to the body, the investigators showed that irradiation localized to the head was sufficient to reproduce the successful ACT therapy achieved with WBI conditioning. Thus, the requirements for irradiation conditioning may vary based on the site of the tumor, in particular in the brain where the mechanisms for recruitment of activated T cells are unique (37).
Here, we have demonstrated that irradiation of the brain and TME is not required to promote successful ACT therapy of autochthonous brain tumors. This discrepancy between our results and those of Plautz et al. in regard to the requirement for local or systemic irradiation may be explained by the choice of donor cells for ACT therapy. Plautz et al. utilized polyclonal lymphocytes, isolated from the tumor-draining lymph node of tumor-bearing donor mice and further activated \textit{in vitro} with bacterial superantigen prior to ACT. The use of activated donor T cells may preclude the requirement for systemic irradiation, as activated donor T cells may traffic directly to the tumor site without prior activation in the lymph nodes. Furthermore, local irradiation has been shown to synergize with ACT of activated donor T cells to promote donor cell accumulation at the tumor site (10). In the current study, we utilized naïve TCR-transgenic CD8$^+$ T cells for ACT therapy. Naïve donor T cells must be activated in the tumor-bearing host prior to infiltration of the tumor, a requirement that may be wholly or partially obviated with the use of activated donor T cells. It follows that systemic lymphodepletion, which favors donor T cell activation and accumulation, may be required to facilitate ACT-mediated tumor regression when naïve donor T cells are used. This notion is supported by the finding that BI conditioning is sufficient to substitute for WBI and promote successful ACT therapy in the current study.

Irradiation of the head and brain, on the other hand, may potentiate the local inflammatory environment and increase tumor cell susceptibility to immune-mediated cytotoxicity, enhancing the efficacy of ACT whether using naïve or activated T cells. While the use of activated TCR-IV donor T cells was not investigated in SV11 mice, it is possible that the requirement for systemic irradiation conditioning can be overcome by using activated donor T cells. Therefore, the differential requirements for local and systemic irradiation conditioning may be predicated by the choice of naïve versus activated donor T cells for ACT therapy.

Despite a lack of observable tumor regression, mice receiving local irradiation plus ACT therapy survived significantly longer than mice treated with either agent alone. Direct irradiation
of tumor cells can enhance anti-tumor immunity through induction of immunogenic cell death, an apoptotic process that promotes tumor antigen uptake and presentation by APCs (38).

Interestingly, irradiation localized to the head was not sufficient to promote early donor T cell accumulation in the brain, despite significant accumulation in the tumor-draining lymph node. Conversely, others have reported increased accumulation of tumor-reactive T cells in the tumor and tumor-draining lymph node following local irradiation, albeit at higher doses of irradiation (9). Indeed, 15 Gy local irradiation induces type I IFN production, and local delivery of type I IFN can substitute for irradiation to mediate immune cell infiltration and tumor regression (39). Recruitment of activated T cells to the brain requires expression of the appropriate chemokines and adhesion molecules (37). Irradiation-induced type I IFN initiates a cascade of immune events, including myeloid cell recruitment, chemokine production, and T cell recruitment to the tumor site (11,39); it also potentiates T cell effector function (11). Adhesion molecule expression by tumor vasculature is increased in a dose-dependent manner following local irradiation (10,40). Thus, a higher dose of local irradiation that induces greater type I IFN production may succeed in promoting enhanced donor T cell recruitment in SV11 mice. Alternatively, donor T cell accumulation in the brain may be primarily dependent on systemic factors induced by BI conditioning. Systemic irradiation increases inflammatory cytokine levels and induces activation of innate immunity, including DCs (8). Activated DCs present tumor antigens and express costimulatory molecules critical to donor T cell activation and differentiation. The differentiation state of donor T cells can have significant impact on donor T cell proliferation, accumulation at the tumor site, and therapeutic efficacy (41), and may represent the dominant determinant of donor cell accumulation in the brain, regardless of local irradiation.

An alternative explanation for prolonged survival of mice receiving local irradiation plus ACT therapy is that local irradiation of the brain may also promote more potent donor T cell activation and a more robust anti-tumor response. T cell responses in the brain are initiated by
DCs that migrate from the brain to the cLN to present antigen to T cells (42). DC migration from the brain to the cLN is enhanced by type I IFN production in the brain (43). The finding that donor T cell accumulation is enhanced in the cLN following local irradiation suggests an increase in the migration of activated DCs to the cLN. However, phenotype analysis on donor T cells in the cLN did not suggest any differences in activation state. These findings imply that the therapeutic benefit of ACT therapy with or without local irradiation is the result of a tumoristatic mechanism mediated by donor T cells. Local irradiation may ultimately promote enhanced donor T cell differentiation or prolonged accumulation in the brain resulting in extended survival in this group; however, additional investigation will be required to define these mechanisms.

These findings have important implications for ACT therapy of cancer. We show that the timing of ACT following lymphodepleting host-conditioning can significantly influence the long-term therapeutic benefit of ACT therapy, including protection from tumor recurrence, which has not been shown previously. While ACT early after host-conditioning was required to maximize therapeutic benefit, we also show that ACT can be delayed following host-conditioning and still mediate highly significant survival benefits. This flexibility in the WBI+ACT therapeutic protocol may benefit patients unable to receive host-conditioning and ACT in immediate succession for health or logistical reasons.

We also show that ACT early after WBI conditioning was required to achieve maximum therapeutic benefit using naïve donor T cells. Current ACT therapeutic protocols in clinical trials utilize ex vivo-expanded, highly-differentiated donor T cells, which may differ from naïve/less-differentiated donor T cells in their response to ACT into a lymphopenic host. Indeed, the differentiation state of tumor-specific donor T cells at the time of ACT has been shown to correlate with therapeutic efficacy and persistence in vivo (41), which may reflect a differential response to the lymphopenic environment. In the days following lymphodepletion, the production of homeostatic cytokines by host stromal cells increases to promote HPE and recovery from
lymphopenia (32). Early ACT at the onset of this surge excels in promoting the therapeutic efficacy of naïve donor T cells, however, ACT at the peak of this surge may benefit activated donor T cells, which express high levels of cytokine receptors and may require more immediate stimulation (41,44). Thus, ACT therapy using activated donor T cells may benefit from a delay in ACT following host-conditioning, as has been suggested by others (35). Future investigation is warranted to determine the influence of the timing of ACT using activated donor cells, as these are used in current clinical trials. Collectively, these data suggest that the timing of ACT following lymphodepleting host-conditioning can have a profound impact on the therapeutic efficacy of ACT therapy of cancer and should be considered during design of therapeutic ACT protocols.

We show that ACT therapy is effective in the absence of direct irradiation of the tumor site. This finding demonstrates that only select host tissues need be irradiated prior to ACT therapy. Here we have demonstrated successful ACT therapy of established brain tumors without irradiating the head – a finding that has important clinical implications, as unnecessary exposure of this vital tissue should be avoided whenever possible (45). It is possible that irradiation conditioning could be further restricted to even fewer tissues in this model. Currently, ACT therapy results in cure in a fraction of treated patients; ongoing investigation and application of ACT therapies to more patients will undoubtedly result in additional cures. It is important, therefore, to take into account the potential long-term side effects of ACT therapy, particularly the use of WBI. Future investigation will be important to define the essential tissues to be irradiated as conditioning for ACT therapy. Ideally the use of image-guided radiation therapy will allow for targeted irradiation that minimizes exposure of adjacent tissues and the potential for off-target effects. Collectively, these findings demonstrate flexibility in the WBI+ACT therapeutic protocol with regard to the tissues irradiated and the timing of ACT following host-conditioning,
and suggest that maximum benefit from ACT therapy is achieved when host-conditioning is optimized to promote brisk, robust donor T cell accumulation and persistence.

References


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Figure 3-1 Delay of ACT by up to seven days post-WBI does not compromise acute tumor regression on day +10.  

a, Schematic illustrating experimental design. All mice received WBI conditioning at approximately 80 days of age.  
b, Representative H&E stained brain sections show evidence of stromal condensation (dashed line) in mice from WBI(day -10)+ACT, WBI(day -7)+ACT, and WBI(day -1)+ACT treatment groups on day +10 (100x magnification).  
c, The largest cross-sectional area of tumor from each mouse brain was measured and is plotted.  
d, The percentage of mice in each group with evidence of residual brain tumor on day +10 is plotted.  

T, tumor; ns, not significant
Figure 3-1

(a) Lesion area (mm$^2$)

Day: -10 -7 -4 -1 0 +10

- WBI(day -10)+ACT: WBI +ACT Analysis
- WBI(day -7)+ACT: WBI +ACT Analysis
- WBI(day -4)+ACT: WBI +ACT Analysis
- WBI(day -1)+ACT: WBI +ACT Analysis

(b) WBI(day -10)+ACT
WBI(day -7)+ACT
WBI(day -1)+ACT

(c) ns ns ns

(d) % of mice with residual lesion
Figure 3-2 Delay of ACT following WBI does not compromise donor T cell accumulation on day +10.  

a, Representative dot plots showing tetramer-IV x CD8 staining on gated live cells from spleens.  
b, The percent tetramer-IV+ of total live cells in spleens is plotted for all mice.  
c, The total number of tetramer-IV+ cells in spleens is plotted for all mice.  
d, Flow cytometry was performed on brain samples from representative mice from each treatment group. Dot plots show tetramer-IV x CD8 staining gated on live cells in the brain.  
e, Representative dot plots showing CD44 x CD62L staining gated on tetramer-IV+ cells in spleens and brains.
Figure 3-2
Figure 3-3 Delay of ACT following WBI does not compromise tumor elimination and donor T cell persistence. 

*a*, Representative H&E stained brain sections from mice in each treatment group are shown at day +30 (40x magnification).  

*b*, Representative dot plots show tetramer-IV x CD8 staining gated on live cells from spleen and cLN on day +30.  

*c*, The percentage and total number of tetramer-IV⁺ cells for each mouse are plotted.  

ns, not significant
Figure 3-3
Figure 3-4 ACT early after WBI conditioning is required to maximize therapeutic benefit. Groups of SV11 mice were left untreated or received the indicated treatment and were monitored for tumor recurrence. 

- **a**, The percentage of surviving mice versus age is plotted.
- **b**, Statistical differences in survival were calculated using the log-rank test. Data are pooled from multiple experiments.
- **c**, Two mice in the WBI(day -7)+ACT group were euthanized at 212 days of age due to advanced tumor progression and analyzed by tetramer-IV x CD8 staining to detect persistent donor T cells in the brain. Plots showing tetramer-IV x CD8 staining are gated on live cells in the brain. Plots showing IFNγ x CD8 staining on brain cells following incubation with site IV peptide or control peptide and intracellular cytokine staining.
**Figure 3-4**

(a) Graph showing percent survival over days of age for different groups: WBI only, WBI (day -1) + ACT, WBI (day -7) + ACT, Untreated. The graph includes a legend indicating untreated, WBI only, and treated groups.

(b) Table summarizing group data and statistical significance:

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBI (day -1) + ACT</td>
<td>306</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBI (day -7) + ACT</td>
<td>212</td>
<td></td>
</tr>
<tr>
<td>WBI (day -1) + ACT</td>
<td>306</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Untreated</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>WBI (day -7) + ACT</td>
<td>212</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Untreated</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>WBI only</td>
<td>115</td>
<td>0.5821</td>
</tr>
<tr>
<td>Untreated</td>
<td>111</td>
<td></td>
</tr>
</tbody>
</table>

(c) Images showing flow cytometry data for different peptides and cytokines in mouse brain. The images indicate CD8+ cell counts and fluorescence intensity for specific peptides and cytokines.
Figure 3-5 Initial proliferation of donor T cells is not impacted by WBI conditioning. 

a, Representative dot plots show tetramer-IV x CD8 staining gated on live cells from spleens on day +2. Corresponding histograms illustrate CFSE dilution in tetramer-IV⁺ donor T cells gated as shown. 
b, The percentage of undivided donor T cells in spleens on day +2 is plotted. 
****p<0.0001
Figure 3-5

(a) Spleen

(b) % Undivided of Tetramer + T cells

- WBI: day -1, none, day -7, day -1
- Mice: B6 (TA g) SV11
- ACT: + + + +

**** ns ns
Figure 3-6 Early donor T cell accumulation in the spleen is impaired following delayed ACT. 

a, Representative dotplots show tetramer-IV x CD8 staining gated on live cells and CD44 x CD62L staining gated on tetramer-IV⁺ cells in the spleen on day +5. 
b, The percentage of tetramer-IV⁺ cells in the spleen on day +5 is graphed. 
c, The total number of tetramer-IV⁺ cells in the spleen on day +5 is graphed. 
d, Total cell counts from spleens are graphed. *p<0.05; ns, not significant
Figure 3-6
Figure 3-7 Early donor T cell accumulation in the brain is impaired following delayed ACT. 

a. Representative dotplots show tetramer-IV x CD8 staining gated on live cells and CD44 x CD62L staining gated on tetramer-IV⁺ cells in the brain on day +5. b. The percentage of tetramer-IV⁺ cells in the brain on day +5 is graphed. c. The total number of tetramer-IV⁺ cells in the brain on day +5 is graphed. d. Total cell counts from percoll-enriched cell fractions from brains are graphed. *p<0.05; ns, not significant
Figure 3-7
Figure 3-8 Delay of ACT following WBI conditioning reduces antigen-independent homeostatic proliferation of donor T cells. a, Representative CD44 x CD62L staining on donor T cells undergoing homeostatic proliferation is shown. b, Corresponding histograms illustrate CFSE dilution in tetramer-gB\(^+\) donor T cells (filled histogram). Unlabeled tetramer-gB\(^-\) CD8\(^+\) T cells are shown for reference (empty histogram). c, The CFSE mean fluorescence intensity (MFI) of TCR-gB donor T cells on day +5 from spleen and cLN is graphed.
Figure 3-8
Figure 3-9 Local irradiation conditioning focused to the body is sufficient to promote rapid accumulation of donor T cells in the lymphoid organs and at the tumor site on day +5. Groups of SV11 mice received either no conditioning, HI, or BI conditioning on day -1. All mice received TCR-IV ACT on day 0. Five days later, flow cytometry was performed on spleens, cLNs, and brains. 


*a*, Illustration depicting the irradiation field and target tissues irradiated in each treatment group. 


*b-c*, Graphs showing total cell counts from spleen and cLN of mice in all groups. 


d, Representative plots are gated on CD45.2⁺ cells and show tetramer-IV x CD8 staining with frequency of TCR-IV cells indicated. 


e-g, Bar graphs show the percent tetramer-IV⁺ cells (mean±SEM) of total CD45.2⁺ cells. 


*h-j*, The total number of tetramer-IV⁺ T cells is plotted. n=6 mice/group. Data is pooled from two independent experiments. Statistical significance was determined using Student’s t-test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant
Figure 3-9
Figure 3-10 Irradiation focused to the body promotes enhanced effector-differentiation of donor T cells in the lymphoid organs and at the tumor site on day +5. Groups of SV11 mice received either no conditioning, HI, or BI conditioning on day -1. All mice received TCR-IV ACT on day 0. Five days later, flow cytometry was performed on spleens, cLNs, and brains. a-c, Histograms illustrate expression of phenotypic markers from representative mice gated on either CD8{Tetramer}{IV}+ cells (dark line) or CD8{Tetramer}{IV}− cells (filled gray histogram). d-f, Bar graphs show the frequency (mean±SEM) of CD44hiCD62Llo effector cells within the tetramer-IV+ population. n=6 mice/group. Data is pooled from two independent experiments. Statistical significance was determined using Student’s t-test. *p < 0.05; **p < 0.01; ****p < 0.0001; ns, not significant
Figure 3-10
Figure 3-11 Irradiation conditioning focused to the body is sufficient to promote enhance accumulation of donor T cells in the spleen and cLN on day +10. Groups of SV11 mice received either no conditioning or HI or BI conditioning on day -1. All mice received TCR-IV ACT on day 0. Ten days later, flow cytometry was performed on spleens and cLNs, and brains were fixed, sectioned, and H&E stained. a-b, The total number of tetramer-IV$^+$ T cells was calculated and plotted. c-d, Histograms illustrate expression of phenotypic markers from representative mice gated on either CD8$^+$tetramer-IV$^+$ cells (dark line) or CD8$^+$tetramer-IV$^-$ cells (filled gray histogram). e-f, Bar graphs show the frequency (mean±SEM) of CD44$^{hi}$CD62L$^{lo}$ effector cells within the tetramer-IV$^+$ population. Data is pooled from two independent experiments. n=5-6 mice/group. Statistical significance was determined using Student’s $t$-test. *$p$ < 0.05; **$p$ < 0.01; ***$p$ < 0.001; ****$p$ < 0.0001; ns, not significant
Figure 3-11
Figure 3-12 Irradiation conditioning focused to the body is sufficient to promote ACT-mediated acute tumor regression independently of irradiation of the tumor. Groups of SV11 mice received either no conditioning or HI or BI conditioning on day -1. All mice received TCR-IV ACT on day 0. Ten days later, flow cytometry was performed on spleens and cLNs, and brains were fixed, sectioned, and H&E stained. a, Representative sections from each mouse are shown. b, The largest cross-sectional area of tumor from each mouse is plotted. Open symbols indicate observation of stromal condensation within the lesion. Statistical significance determined using the Mann Whitney test. Data is pooled from two independent experiments. n=5-6 mice/group.
Figure 3-12
Figure 3-13 Irradiation conditioning focused to the body promotes extended ACT-mediated survival in association with donor T cell persistence in the lymphoid organs and at the tumor site. Groups of SV11 mice received the indicated conditioning with or without TCR-IV ACT and were monitored for tumor recurrence. a, The percentage of surviving mice versus age is plotted. b, Statistical differences in survival were calculated using the log-rank test. Data are pooled from multiple experiments with a total of at least 7 mice/group. c, Mice in the BI+ACT group were sacrificed at 235 days of age and analyzed by tetramer-IV x CD8 staining to detect persistent TCR-IV T cells. Plots showing tetramer-IV+ cells gated on total CD45.2+ cells in the spleen, cLN, and brain are shown from one representative mouse. Values indicate mean±SEM (n=6).
Figure 3-13
Chapter 4

Protection from tumor recurrence following adoptive immunotherapy varies with host conditioning regimen despite initial regression of autochthonous murine brain tumors

Abstract

Adoptive T cell transfer (ACT) has achieved clinical success in treating established cancer, particularly in combination with lymphodepleting regimens. Our group previously demonstrated that ACT following whole-body irradiation (WBI) promotes high-level donor T cell accumulation, regression of established brain tumors, and long-term protection from tumor recurrence in a mouse model of SV40 T antigen-induced choroid plexus tumors. Here we asked whether an approach that can promote strong donor T cell responses in the absence of WBI might also produce this dramatic and durable tumor elimination following ACT. Agonist anti-CD40 antibody can enhance antigen-specific CD8+ T-cell responses and has shown clinical efficacy as a monotherapy in the setting of cancer. We show that anti-CD40 conditioning promotes rapid accumulation of tumor-specific donor CD8+ T cells in the brain and regression of autochthonous T antigen-induced choroid plexus tumors, similar to WBI. Despite a significant increase in the lifespan, tumors eventually recurred in anti-CD40-conditioned mice coincident with loss of T cell persistence from both the brain and lymphoid organs. T cells activated in anti-CD40-conditioned mice could be rescued by transfer into irradiated recipients, indicating that T cells were not irreversibly programmed to die by anti-CD40-conditioning. Depletion of CD8+ T cells from the peripheral lymphoid organs of WBI-conditioned recipients failed to promote tumor recurrence, but donor cells persisted in the brains long-term in CD8-depleted mice. These results demonstrate
that anti-CD40 conditioning effectively enhances ACT-mediated acute elimination of autochthonous tumors, but suggest that mechanisms associated with WBI conditioning, such as the induction of long-lived T cells, may be critical for protection from tumor recurrence.

Introduction

Adoptive T-cell transfer (ACT) with CD8$^+$ T cells has shown promise as a therapy for solid tumors, including metastases. The use of host-conditioning regimens such as non-myeloablative chemotherapy and whole-body irradiation (WBI) prior to ACT has increased overall response rates to an impressive 50% or higher and improved response durability (1,2). WBI has broad systemic effects, a subset of which are thought to be critical for therapeutic success of ACT (3-6). Although predictors of therapeutic outcome remain elusive, certain “naïve-like” characteristics of donor T cells, such as increased telomere length and CD27 expression, correlate with success (7). Additionally, long-term persistence of T cells following ACT has been associated with complete, durable remissions in clinical trials (2). A current challenge is to broaden the applicability of ACT-based therapies, which require large numbers of *ex vivo* expanded T cells and are targeted to select patients.

WBI-conditioning was shown previously to enhance ACT in mice that develop autochthonous tumors due to transgenic expression of the simian virus 40 (SV40) large T antigen (T Ag) oncoprotein within unique tissues (8-11). In particular, WBI facilitates rapid and high-level accumulation of adoptively transferred T cells in the brains of SV11 mice bearing choroid plexus tumors (9,10,12). Line SV11 mice express T Ag from the SV40 promoter, which selectively targets high-level oncoprotein expression in the choroid plexus of the brain and low levels in the kidney, although tumor formation is restricted to the choroid plexus (13). T Ag
expression in the choroid plexus begins within 14 days of birth and results in the appearance of microscopic papillomas by 35 days (14). Tumors progress rapidly beginning at approximately 80 days of age, causing death at a mean age of 105 days (14,15). Due to low-level transgene expression in the thymus (unpublished observations), SV11 mice are immunologically tolerant to T Ag and unable to mount a CD8$^+$ T-cell response toward the dominant T Ag determinants, including the immunodominant site IV determinant (residues 404-411) (8). However, transfer of T Ag-specific donor CD8$^+$ T cells into 80 day-old WBI-conditioned mice results in rapid, high-level T-cell accumulation within the brain, tumor elimination, T-cell persistence at the tumor site, and prevention of tumor recurrence (10). These results raise the question of whether alternative approaches that trigger high-level T-cell accumulation at the tumor site can promote regression of autochthonous tumors, independent of the additional mechanisms associated with irradiation.

Agonist anti-CD40 antibodies promote strong anti-tumor CD8$^+$ T-cell responses in vivo (16-19). A member of the tumor necrosis factor-receptor superfamily, CD40 is expressed on the surface of professional antigen-presenting cells (pAPC), as well as endothelial cells and some tumors (20). Ligation with CD40 ligand (CD154), expressed by CD4$^+$ T cells, results in the upregulation of major histocompatibility complex class II and costimulatory molecules on pAPCs and licenses these cells to trigger productive CD8$^+$ T-cell activation and differentiation (21-23). CD40 agonists mimic this signal and promote anti-tumor responses through mechanisms including induction of anti-tumor T-cell responses (16,24), recruitment of tumoricidal myeloid cells (25), activation of tumor vasculature (26), and direct cytotoxicity of CD40-expressing tumors (27). In clinical trials, anti-CD40 administration has resulted in objective responses (28), and this cancer immunotherapeutic agent is prioritized for investigation by the National Cancer Institute-supported Cancer Immunotherapy Trials Network (29). The combination of anti-CD40 conditioning with other immune-based therapies has the potential to produce more significant anti-tumor effects (30). In particular, combination with ACT has yet to be translated to human
cancer patients. Anti-CD40 conditioning promotes the *in vivo* expansion of adoptively transferred T cells capable of controlling solid tumor progression in experimental models (18,31-33), however the effects on immune surveillance and tumor recurrence have not been thoroughly investigated. Thus, anti-CD40 conditioning could potentially broaden the use of ACT therapy to cancer patients for whom lymphodepleting chemotherapy or WBI is contraindicated.

In the current study, we directly compared the therapeutic and immunological impact of WBI and anti-CD40 conditioning on ACT-mediated immunotherapy of autochthonous brain tumors. We show that anti-CD40 reproduced the initial T-cell accumulation and dramatic tumor elimination observed in WBI-conditioned mice while also significantly extending survival. However, WBI was superior in establishing both donor T-cell persistence and protection from tumor recurrence at late time points.

**Materials and Methods**

**Mice**

SV11 mice (34) (C57BL/6-Tg(TAg)11Bri) were maintained as previously described (8) and used at 75-85 days of age in all experiments. B6.Cg-Tg(TcraY4,TcrbY4)2025Tdsc, or TCR-IV, mice express a T cell receptor (TCR) αβ pair specific for the H2-K\(^b\) restricted site IV epitope (10). For some experiments, TCR-IV males were bred with B6.PL-Thy1\(^a\)/CyJ (CD90.1) homozygous females to yield CD90.1\(^+\) donor cells. All mice were maintained in specific pathogen-free conditions at the Milton S. Hershey Medical Center animal facility. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Penn State Hershey College of Medicine.
**Host conditioning and adoptive T cell transfer**

Irradiation-conditioned mice were administered 4 Gy WBI on day -1 using a $^{60}$Co Gammacell irradiator (Nordion International) or an X-RAD 320ix biological x-ray irradiator (Precision X-Ray Inc.). Anti-CD40- and control-conditioned mice received 100 µg purified anti-CD40 (clone FGK45, BioXcell) or control rat IgG (Sigma) on days -1 and +1 by intraperitoneal (i.p.) injection. Whole-cell populations were recovered from spleens and axillary, brachial, superficial cervical, mesenteric, inguinal, and lumbar lymph nodes (35) of TCR-IV donor mice. CD8$^+$ cells were enriched by autoMACS magnetic sorting using the manufacturer’s recommendations (Miltenyi Biotec), resulting in approximately 90% pure CD8$^+$ TCR-IV$^+$ T cells. 1 x 10$^6$ naïve TCR-IV T cells were administered intravenously in 200 µL PBS on day 0 of the experiments. For adoptive transfer into secondary recipients (Fig. 4-8 and 4-9), RBC-depleted splenocytes recovered from primary SV11 recipients were CD90.1$^-$ enriched by magnetic sorting, yielding 75-85% CD90.1$^+$ TCR-IV$^+$ T cells. 1 x 10$^6$ TCR-IV T cells were then administered intravenously.

**Lymphocyte isolation and flow cytometric analysis**

On the day of analysis, spleens, superficial cervical lymph nodes (cLN), and brains were removed from CO$_2$-euthanized mice following exsanguination. Lymphocytes from spleens and cLN were obtained as previously described (8). Briefly, spleens and cLN were mechanically disrupted by pressing tissue through metal screens to obtain single-cell suspensions in approximately 7 mL cold RPMI-1640 (henceforth called RPMI) with GlutaMAX™ (Gibco) supplemented with 2% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 10 mM HEPES, and 25 µg/mL pyruvic acid. Red blood cells (RBCs) were eliminated by 5 minute incubation in warm tris ammonium chloride. Following RBC-
depletion, white cells were washed once in cold RPMI, then resuspended in 1-5 mL cold RPMI for live cell counting by trypan blue exclusion. Prior to removing spleen and cLN cell aliquots for antibody staining, samples were left on ice for 5 minutes to allow large debris to settle. Lymphocytes from thymi were obtained using the protocol for spleen/cLN lymphocyte isolation. Brain lymphocytes were obtained as previously described (9). Briefly, brains were minced with a razorblade and mixed by pipetting in cold RPMI. Dispersed cells were placed on ice and large debris allowed to settle twice for 5 minutes, after which the supernatant containing cells was collected. The single-cell suspension was RBC-depleted and washed before centrifugation (500 x g for 20 minutes) on a density gradient (Percoll, 70%:32%). Cells at the interface were removed with a Pasteur pipet, washed, and live cells counted by trypan blue exclusion. Lymphocytes from kidneys were obtained using the protocol for brain lymphocyte isolation. Lymphocytes from bone marrow were obtained from one femur/mouse. Both ends of the femur were removed with scissors and cold media was pipetted through to flush out bone marrow cells. Bone marrow cells were washed in media before flow cytometry staining. Fluorochrome-labeled antibodies were obtained from eBioscience (CD8α clone 53-6.7, CD90.1 clone HIS51, CD44 clone IM7, killer-cell lectin like receptor G1 (KLRG1) clone 2F1), BD Biosciences (CD45.2 clone 104, CD127 clone SB/199, TCRβ clone H57-597), BioLegend (CD8β clone 53-5.8, CD62L clone MEL-14), and Tonbo Biosciences (CD8α clone 53-6.7). Site IV/Kb tetramers were prepared and used to stain site IV-specific T cells as previously described (10,36). Briefly, cells were plated in 200 µL at a density no greater than 1x10^7 cells/mL in RPMI in round-bottom, tissue culture-treated 96-well plates. Plated cells were washed twice with 150 µL cold FACS buffer (PBS + 2% FBS + 0.125 mM sodium azide). After each wash, cells were centrifuged (1400 rpm, 2 minutes, 4°C), and supernatant was removed by swiftly overturning the plate and blotting face down on a clean paper towel. Blocking was performed in 50 µL cold FACS buffer containing Fc at a 1:100 dilution (10 minutes, room temperature). Following blocking, cells were washed 1 x 150 µL cold
FACS buffer before antibody/tetramer staining. Surface staining with antibodies and tetramers was performed simultaneously in 50 µL cold FACS buffer (15 minutes, room temperature, protected from light). Antibodies and tetramers were used at a 1:100 dilution. Following staining, cells were washed 3 x 150 µL FACS buffer and resuspended in 200-300 µL cold FACS buffer for flow cytometry. Data were acquired using an LSR II SORP, LSRFortessa or FACSCanto II (BD Biosciences) flow cytometer in the Penn State Hershey Flow Cytometry Core Facility. Data analyses were performed with FlowJo software (TreeStar Inc.).

**Intracellular cytokine staining and degranulation assay**

Isolated lymphocytes were incubated with T Ag site IV 404-411 peptide variant C411L (VVYDFLK) or control herpes simplex glycoprotein B 498-505 peptide (SSIEFARL) for 5-6 hours at 37°C in the presence of Brefeldin A (cytokine staining; Sigma) or Brefeldin A and fluorescein isothiocyanate (FITC)-conjugated CD107a antibody (degranulation assay, clone 1D4B; BD Biosciences). Following incubation, cells were stained for surface markers as described above, then fixed and permeabilized using Cytofix/Cytoperm (BD Pharmingen). For cytokine staining, cells were subsequently incubated with fluorochrome-conjugated antibodies for 15 min at room temperature, washed 3 times, and analyzed by flow cytometry. Fluorochrome-conjugated antibodies were obtained from eBioscience (interferon (IFN)γ clone XMG1.2, TNFα clone MP6-XT22, IL-2 clone JES6-5H4).

**Survival analysis**

Median lifespan was determined by monitoring mice for the development hydrocephalus and neurological signs indicative of advanced tumor development such as lethargy and ataxia (8).
Symptomatic mice were euthanized and Kaplan-Meyer survival curves were created using GraphPad Prism software (GraphPad Prism Software, Inc.).

**Histology and immunohistochemistry**

Following sacrifice, mice were perfused with PBS followed by 10% neutral buffered formalin (NBF). Brains from perfused mice were removed and stored overnight in NBF and then transferred to 70% ethanol. Fixed brains were paraffin embedded and representative coronal sections were collected throughout the brain. Brain sections were hematoxylin and eosin (H&E) stained and the maximum tumor diameter was determined for each mouse by light microscopy. TAg immunohistochemistry (clones pAB901 and pAB419) was performed as described (37). Images were captured using an Olympus BX51 microscope with a 2x, 20x, or 40x objective fitted with an Olympus DP71 digital camera and cellSens Standard 1.6 imaging software (Olympus). Histological sections were evaluated blindly by a board certified veterinary pathologist.

**Anti-CD8 antibody production and in vivo T cell depletion**

Anti-CD8 monoclonal antibody clone 2.43 (38) was produced in BD Cell MAb animal component-free medium (BD Biosciences) using CELLine reactor flasks (Corning) per the manufacturer’s recommendations. Concentrated antibodies were dialyzed into PBS, purity-verified by gel electrophoresis and aliquots stored at -20°C. Beginning on day +20 of experiments, mice received weekly i.p. injection of 100 µg of control antibody (rat IgG; Sigma) or anti-CD8 antibody in 200 µL PBS.
Statistics

All statistical tests were performed using GraphPad Prism software. Unpaired Student’s $t$-test was used to determine significance unless otherwise noted. A $p$-value less than 0.05 was considered statistically significant and is indicated using *, **, ***, **** ($p < 0.05, p < 0.01, p < 0.001, p < 0.0001$, respectively).

Results

*Host conditioning with anti-CD40 induces high-level T cell accumulation in the lymphoid organs and brains of SV11 mice at early time points.*

We initiated experiments at 80 days of age, when choroid plexus tumors fully or partially fill the ventricles but typically are not invasive (9). Previously, WBI conditioning was found to accelerate accumulation of donor T cells within the brains of SV11 mice, which first appeared on day +5 following ACT (10). We first compared the magnitude and kinetics of donor T-cell accumulation in SV11 mice conditioned with anti-CD40 agonist antibody or sub-lethal WBI followed by ACT with naïve CD8$^+$ TCR-IV T cells that are reactive with the immunodominant H-2K$^b$-restricted site IV determinant of T Ag. We utilized naïve donor T cells in order to evaluate the impact of each conditioning regimen on initial T-cell activation, differentiation, and accumulation. While current clinical protocols utilize *ex vivo* expanded T cells for ACT, studies in mice have demonstrated improved efficacy of less-differentiated donor cells for ACT-based therapies (39), resulting in efforts to generate donor T cells with a younger phenotype (40,41).

As early as day +4 post-ACT, increased frequencies of TCR-IV T cells were detected in the spleens of mice that received anti-CD40 or WBI relative to unconditioned mice (Fig. 4-1a) and these proportions further increased by day +5. Similar results were observed in the tumor-
draining cLNs (unpublished observations). Despite the lower frequency of TCR-IV T cells detected in the spleens of anti-CD40-conditioned mice, the absolute number of TCR-IV T cells was approximately 10-fold higher than in WBI-conditioned mice by day +5 (Fig. 4-1b). Total splenocyte counts revealed a general increase in cellularity in anti-CD40-conditioned mice, in contrast to lymphodepletion in WBI-conditioned mice (Fig. 4-1c). These results demonstrate that anti-CD40 dramatically increases donor T-cell accumulation in the lymphoid organs of SV11 mice.

Consistent with previous results (10), high-level T-cell accumulation in the brain was first detected on day +5 post-ACT in mice that received WBI conditioning (Fig. 4-1d), and this effect was duplicated in mice that received anti-CD40. Unconditioned mice accumulated few TCR-IV T cells in the brain at this early time point. The total number of TCR-IV T cells in the brain of anti-CD40-conditioned versus WBI-conditioned mice at day +5 was similar (Fig. 4-1e), indicating that accumulation in the brain is not proportional to accumulation in the periphery. Likewise, total cell accumulation in the brain was similar for WBI- and anti-CD40-conditioned mice (Fig. 4-1f). Thus, both conditioning regimens promote early, high-level TCR-IV T-cell accumulation in the brain of tumor-bearing mice despite their differential effects on T-cell numbers in the lymphoid organs.

**Anti-CD40-enhanced ACT promotes initial regression of established tumors**

We asked whether T-cell accumulation in the brains of anti-CD40-conditioned mice was associated with tumor regression. Groups of mice received anti-CD40, control immunoglobulin (IgG), or WBI conditioning prior to ACT and were sacrificed on day +10 to assess tumor burden. Nine of ten mice in the anti-CD40-conditioned group showed evidence of tumor regression, either lacking detectable tumors or having only residual small lesions that displayed stromal
condensation (Fig. 4-2a-b). All mice that received ACT with WBI conditioning had either small residual lesions or none at all, consistent with previous observations (10). In contrast, tumors were detected in 100% of control IgG-conditioned mice, which in some cases invaded into the brain parenchyma (Fig. 4-2a-b).

**Donor T cells contract dramatically in anti-CD40-conditioned SV11 mice**

We evaluated T-cell accumulation on days +6 and +10 post-ACT to determine the kinetics of the T-cell response following recruitment into the brain on day +5. On day +6, TCR-IV T cells comprised a high percentage of CD8$^+$ T cells in the spleens and brains of anti-CD40- and WBI-conditioned, but not control IgG-conditioned mice (Fig. 4-3a-b). Donor cell frequencies were reduced in the spleen by day +10, but the reduction was much more dramatic in anti-CD40-conditioned mice where T-cell frequencies dropped to levels observed in control IgG-conditioned mice (Fig. 4-3a). This reduced frequency was paralleled by a 48-fold decrease in total splenic TCR-IV T cells between days +6 and +10 (Fig. 4-3c and e), while the total number of splenic TCR-IV T cells remained constant in WBI-conditioned mice. TCR-IV cell contraction in the brain was less pronounced (Fig. 4-3d and f), with the frequency significantly decreasing only for anti-CD40-conditioned mice (Fig. 4-3b), but with similar numbers of donor T cells persisting by day +10 in both WBI- and anti-CD40-conditioned mice. These results demonstrate that anti-CD40 promotes high-level TCR-IV T-cell accumulation in the lymphoid organs of SV11 mice followed by dramatic contraction by day +10 when tumors have regressed. Contraction was less pronounced in the brain where a significant pool of donor T cells remained at day +10.

Donor T cells were uniformly CD44$^{hi}$CD62L$^{lo}$, indicating that donor cells in all groups had undergone initial T-cell activation and differentiation (Fig. 4-4a). We also found that the proportion of accumulating TCR-IV T cells that expressed the inhibitory receptor KLRG1 was
slightly elevated in spleens and brains of anti-CD40-conditioned mice on day +6 (Fig. 4-4b). By day +10, the KLRG1+ splenic subpopulation appeared elevated in both WBI- and anti-CD40-conditioned mice, but statistical significance was only achieved in anti-CD40-conditioned mice (Fig. 4-4c). Meanwhile, KLRG1 expression in the brain remained low on day +10 in all groups, and was significantly reduced in anti-CD40-conditioned mice (Fig. 4-4c). These results suggest that accumulation of terminally-differentiated or senescent T cells is significantly enhanced by anti-CD40 conditioning.

Anti-CD40-enhanced ACT promotes increased survival but not long-term surveillance against tumor recurrence

Survival of mice that received anti-CD40 conditioning plus TCR-IV ACT was significantly prolonged compared to mice that received TCR-IV ACT with control IgG (Fig. 4-5a-b). However, all mice that received anti-CD40 conditioning plus TCR-IV ACT eventually succumbed to tumor recurrence (median lifespan = 176 days of age). This was in contrast to mice that received WBI conditioning plus TCR-IV ACT, all of which survived until termination of the experiment at 215 days of age and appeared healthy and asymptomatic. A non-invasive tumor was observed in one mouse with papillary to focally solid architecture that did not resemble primary untreated tumors ((14) Fig. 4-5c: Tumor recurrence). TCR-IV T cells were detected in the spleen and cLN of all surviving mice in this group (Fig. 4-5d). Approximately half of the site IV-specific CD8+ T cells in the spleen and cLN retained the ability to produce IFNγ and degranulate in response to specific antigen stimulation (Fig. 4-5d). Thus, ACT promotes initial tumor regression and significantly extends survival in anti-CD40-conditioned mice, but tumors eventually recur in 100% of mice. Conversely, enduring tumor control was observed in WBI-
conditioned mice, which was associated with the persistence of functional TCR-IV T cells for over 130 days.

**Donor T cells fail to persist in anti-CD40-conditioned SV11 mice following acute tumor regression**

We asked whether tumor recurrence in anti-CD40-conditioned mice correlated with loss of persisting TCR-IV T cells. Groups of mice were treated as described in figure 2 using CD90.1⁺ donor TCR-IV T cells. On day +30, TCR-IV T cells were apparent in both spleen and brain of WBI-conditioned mice (Fig. 4-6a-b). In contrast, only low frequencies of TCR-IV T cells were detected in anti-CD40- and control IgG-conditioned mice (Fig. 4-6a-b). Quantitative analysis in the spleen, cLN, and brain reinforced that TCR-IV T cells failed to persist in anti-CD40-conditioned mice (Fig. 4-6c). Within the spleen of WBI-conditioned mice, a subset of the persisting TCR-IV T cells were KLRG1⁺ (Fig. 4-6d), indicative of terminally-differentiated effector cells and suggestive of ongoing immune surveillance. However, TCR-IV T cells persisting in brains from all groups lacked KLRG1 expression (Fig. 4-6e). Persisting TCR-IV T cells in spleens and brains of all mice expressed a similar CD44⁺CD62L⁻ phenotype (Fig. 4-6d-e). These results illustrate that TCR-IV T cells fail to persist at significant levels in the brain and lymphoid organs of anti-CD40-conditioned mice despite their continued presence and signature of ongoing immunity in WBI-conditioned mice.

An alternative explanation for loss of donor T cell persistence in anti-CD40-conditioned mice is emigration of donor T cells out of the tissues analyzed (spleen, cLN, brain). To test this, we also assessed T cell persistence in thymus, bone marrow, and kidney on day +20 post-ACT with CD90.1⁺ TCR-IV T cells in groups of mice that had received conditioning with anti-CD40, control IgG, or WBI. Donor T-cell persistence in spleen, cLN, brain, kidney, and thymus was
significantly higher by percentage in WBI-conditioned mice, consistent with observations at day +30 in spleen, cLN, and brain (Fig. 4-7a-b). Interestingly, TCR-IV T cells comprised a large fraction of CD45.2^+ cells in the kidney of WBI-conditioned mice. While T Ag expression has not been detected at the protein level in the kidney of SV11 mice, mRNA transcripts have been detected previously (unpublished observations, T.D.S.). TCR-IV T cell persistence in thymus and bone marrow was only slightly above background. These results suggest that loss of donor T cells in anti-CD40-conditioned mice was not due to emigration of these cells to other tissues.

**TCR-IV T cells programmed in anti-CD40-conditioned mice are rescued following transfer into WBI-conditioned mice**

We postulated that priming of TCR-IV T cells in anti-CD40-conditioned mice programmed all TCR-IV T cells for terminal differentiation. To test this hypothesis, we re-isolated donor CD90.1^+ TCR-IV T cells from anti-CD40-conditioned SV11 mice on day +4 post-ACT when T cells had begun to accumulate to high levels in the lymphoid organs but not in the brain (Fig. 4-8a). On day +4, sorted TCR-IV T cells included both CD127^KLRG1^+ short-lived effector cell (SLEC) and CD127^KLRG1^- memory-precursor effector cell (MPEC) populations (Fig. 4-8b). As expected, these donor cells were also CD44^hi, and approximately half had down-regulated the CD62L homing receptor (Fig. 4-8b). Recovered TCR-IV T cells were then transferred into SV11 or transgene negative (SV11') mice that had received WBI on day -1 (Fig. 4-8a). T cells were quantified from spleens of representative recipient mice in both groups on day +5 to ensure engraftment (Fig. 4-8c).

To evaluate T-cell persistence following secondary transfer, TCR-IV T-cell accumulation and phenotype were assessed on day +22. TCR-IV T cells primed in anti-CD40-conditioned mice persisted at higher levels in both the lymphoid organs and brain of irradiated SV11 mice.
compared to irradiated transgene-negative mice, indicating that antigen recognition was required for high-level persistence (Fig. 4-8d). TCR-IV T cells recovered from SV11 mice showed a range of CD127 and KLRG1 staining in the spleen, with the SLEC and MPEC populations being inversely proportional in individual mice (Fig. 4-8e), but with all mice having both populations present. In the brain, a uniform CD127 KLRG1´ early-effector cell population predominated (Fig. 4-8e). Taken together, these results show that TCR-IV T cells primed in anti-CD40-conditioned SV11 mice formed a persistent population of tumor-specific T cells following transfer into irradiated SV11 mice. This result suggests that the immune environment rather than initial T-cell programming controls T-cell longevity in tumor-bearing mice.

**TCR-IV T cells programmed in anti-CD40- or WBI-conditioned mice possess similar survival and functional potential following transfer into WBI-conditioned control recipients**

We hypothesized that TCR-IV T cells primed in anti-CD40- or WBI-conditioned mice would possess similar survival and functional potential when transferred into the same environment. To confirm that initial T-cell programming does not determine donor T-cell longevity, we re-isolated donor CD90.1+ TCR-IV T cells from spleens and cLN of mice conditioned with either anti-CD40 or WBI on day +5, a time point when sufficient T-cell accumulation allowed isolation of enough donor T cells, and transferred the isolated TCR-IV T cells into WBI-conditioned SV11´ recipients that had been immunized with a T Ag-expressing cell line on day +1 (Fig. 4-9a). Re-isolated donor T cell pools from anti-CD40- and WBI-conditioned mice expressed similar activated effector phenotypes (Fig. 4-9b). On day +25, tetramer-IV+ donor T cells varied in frequency but were readily detectable in the spleens of all secondary recipients, regardless of whether donor T cells were isolated from anti-CD40- or WBI-conditioned mice (Fig. 4-9c). Additionally, persisting donor T cells possessed similar functional
capacity with regard to the production of IFN\(\gamma\), TNF\(\alpha\), and IL-2 following \textit{in vitro} incubation with site IV peptide (Fig. 4-9d). These results show that donor T cells initially primed in anti-CD40-conditioned mice possess similar survival and functional potential as T cells primed in WBI-conditioned mice following secondary transfer into irradiated control recipients. This suggests that WBI conditioning creates an immune environment that supports ongoing donor T cell persistence and function regardless of initial T cell programming. Furthermore, this signifies host conditioning may be a critical determinant of donor T cell longevity and function long after ACT.

\textit{Sustained peripheral depletion of CD8\(^{+}\) T cells following WBI-enhanced ACT does not promote tumor recurrence or eliminate donor T cells from the brain}

The presence of KLRG1\(^{+}\) TCR-IV T cells at day +30 in the spleens of WBI-conditioned mice suggested that peripheral effector cells may contribute to long-term tumor control. Thus, we depleted CD8\(^{+}\) cells from the lymphoid organs of WBI-conditioned mice that received TCR-IV ACT by weekly injection of anti-CD8 antibody beginning on day +20. Flow cytometric analysis of blood samples showed near complete CD8\(^{+}\) T-cell depletion (Fig. 4-10a-b). Mice were monitored for 12 weeks post-ACT during which time all appeared healthy without symptoms of tumor recurrence. On day +80, mice were sacrificed to assess TCR-IV T-cell levels and tumor burden. Anti-CD8 injections reduced TCR-IV T-cell levels in spleen and cLN, although the latter was not statistically significant due to variation in the control IgG-treated group (Fig. 4-10c). Inspection of brain sections revealed only small lesions in 6/6 mice in the CD8-depleted group (Fig. 4-10d-e). Similar results were obtained in the control-treated group, in which 5/6 mice had only residual lesions and one mouse developed a small mass. T Ag-expressing choroid plexus cells were still detectable in representative sections from both groups (Fig. 4-10f), indicating that
extended survival of WBI-conditioned mice was not due to loss of oncogene expression. The experiment was repeated using CD90.1\(^+\) TCR-IV T cells to evaluate T-cell persistence in the brain. Despite reduced donor-cell levels in the periphery, total numbers of brain-resident TCR-IV T cells were similar between treatment groups (Fig. 4-10g). Thus, long-term depletion of tumor-specific T cells from the circulation of SV11 mice following tumor regression does not promote tumor recurrence or eliminate persisting donor T cells from the brain.

Discussion

Our findings indicate that anti-CD40 conditioning facilitates acute regression of established tumors in combination with ACT but imply that additional immune manipulation is required to establish continuous immune surveillance when tumor recurrence is likely. The combination of anti-CD40 with ACT was previously shown to enhance the magnitude of tumor-specific T-cell responses, but had only a minimal impact on tumor progression (18,31,33,42) unless additional immune modulators, such as interleukin (IL)-2 or immunization, were provided (31-33). Our results demonstrate that agonist anti-CD40 alone not only facilitates rapid, high-level donor CD8\(^+\) T-cell accumulation systemically and at the tumor site but also promotes regression of established autochthonous tumors. This robust response may be explained in part by the choice of target antigen, as targeting the weaker and less stable T Ag site V determinant with this approach previously failed to induce significant regression of choroid plexus tumors unless mice received multiple rounds of immunization (31). Likewise, Cho et al. found that using a combination of anti-CD40, tumor peptide, poly:ICLC, and recombinant IL-2 immune complexes with ACT was most effective against antigenic determinants expressed at relatively high levels on the tumor cells (32). The tumor type and microenvironment may also play a dominant role in the success of the observed response. Anti-CD40-conditioning plus ACT targeting the site I
determinant of T Ag in mice that develop insulinomas induced only a modest delay in tumor progression despite initial T-cell accumulation at the tumor site (18). Whether T cells targeting site IV would promote effective tumor regression in anti-CD40-conditioned mice bearing insulinomas remains to be determined.

Acute TCR-IV T-cell accumulation reached 10-fold higher levels in the spleens of anti-CD40- compared to WBI-conditioned SV11 mice (Fig. 4-1). The basis for this difference could be explained by more efficient antigen presentation or increased support for T-cell proliferation within anti-CD40-conditioned mice, among other explanations. In support of the latter, we found that total cell counts were dramatically increased in the lymphoid organs of anti-CD40-conditioned mice relative to control mice (Fig. 4-1), as previously observed (16). Despite such dramatic differences in the periphery, the number of donor T cells that initially accumulated in the brain was similar among anti-CD40- and WBI-conditioned mice, suggesting that only a threshold of activated T cells need be reached in the periphery to achieve a therapeutic level of T cells in the brain.

We demonstrate that anti-CD40-enhanced ACT significantly increased the lifespan of SV11 mice but did not recapitulate the extended immune surveillance against tumor recurrence achieved in WBI-conditioned mice. Thus, tumor regression is not predictive of protection from tumor recurrence (Figs. 4-2 and 4-4). Rather, T-cell persistence observed in WBI-conditioned mice was associated with long-term progression-free survival. Demonstration that tumors do not recur in WBI-conditioned mice following depletion of peripheral CD8+ T cells (Fig. 4-10) suggests that brain-resident TCR-IV T cells, which were resistant to antibody-based depletion, may provide long-term immune surveillance in the setting of ongoing T Ag expression, although this remains to be proven. These results raise the issue of why long-lived CD8+ T cells were not maintained in anti-CD40-conditioned mice despite recruitment of an equivalent number of TCR-
IV T cells into the brain at early time points. Alternatively, protection from tumor recurrence may be T-cell independent once tumors have been eliminated.

Could the high level of donor T-cell accumulation achieved in anti-CD40-conditioned mice promote loss of the responding cells? Secondary transfer experiments revealed that donor T cells primed in anti-CD40-conditioned mice are not committed to deletion and can form long-lived populations in both the brain and the periphery (Fig. 4-8). Additionally, we observed no difference between donor T cells primed in anti-CD40- or WBI-conditioned mice with regard to persistence and cytokine production following secondary transfer into irradiated control recipients (Fig. 4-9). However, these cells may be subjected to sustained high-level activation if they remain in the environment of anti-CD40-conditioned mice. Of note, deletion of endogenous T cells was observed in human patients that received multiple doses of anti-CD40 (43). The authors suggested that frequent dosing of anti-CD40 may lead to T-cell hyperstimulation and apoptosis. This conclusion is consistent with a study showing that continuous provision of agonist anti-CD40 can suppress the induction of collagen-induced arthritis in mice (44). However, suppression was achieved only if anti-CD40 was provided after collagen administration. Thus, altering the timing of anti-CD40 administration relative to ACT could potentially improve the durability of anti-tumor immune surveillance.

Kedl and coworkers previously showed that anti-CD40 as a monotherapy promoted early deletion of endogenous tumor-specific T cells (42) but that T cells were protected from deletion by immunization with antigen-expressing vaccinia virus. Additionally, provision of toll-like receptor ligands has been shown to enhance the development of T-cell memory in anti-CD40-conditioned mice in vaccine and tumor models (45,46), perhaps through induction of type I IFN which improves responses to survival cytokines such as IL-7 and IL-15 (47). Notably, microbial products such as lipopolysaccharide can translocate across the gut lumen following WBI-conditioning (6), triggering innate immune cells that may contribute to T-cell persistence.
Recently, Zhang et al. demonstrated that IL-15Rα is upregulated on multiple cell types including CD8⁺ T cells, dendritic cells, and B cells following anti-CD40 administration (48). Provision of exogenous IL-15, important for memory T-cell survival (49), was required for successful control of transplantable prostate tumors. This finding raises the possibility that in the current study, limited IL-15 precludes TCR-IV T-cell persistence. Indeed, anti-CD40 promotes the systemic expansion of multiple immune cell types, which may increase competition for survival cytokines between donor T cells and host immune cells. Meanwhile, the lymphodepleting effects of WBI create an environment in which increased cytokine availability, including IL-15, supports the differentiation and survival of donor T cells (3). The combination of ACT with a cocktail of immune-modulatory agents, including anti-CD40, has been shown to reduce initial tumor burden and promote durable T-cell responses in a melanoma mouse model (32). Taken together, these studies suggest that use of combination therapy in which other immune modulators are included with anti-CD40 may lead to prolonged immune surveillance.

While WBI plus ACT was sufficient to mediate long-term progression-free survival in SV11 mice, immunotherapy against more resistant tumors may benefit from combining WBI with anti-CD40. As demonstrated, WBI provides limited control of tumor progression in the absence of ACT (Fig. 4-5). However, WBI may enhance tumor antigen availability through direct induction of immunogenic tumor cell death. While this mechanism has been primarily investigated using higher doses of irradiation (50-52), a recent study provides evidence that immunogenic cell death may be induced using lower doses of irradiation as used in the current study (53). Therefore, WBI and anti-CD40 could play complementary roles in the setting of ACT, with WBI increasing tumor antigen availability for APCs that become licensed for enhanced T-cell priming by anti-CD40 (54). WBI might also improve T-cell persistence when used in combination with anti-CD40 by increasing donor T-cell access to survival cytokines through lymphodepletion (3). Conversely, combining anti-CD40 with WBI may be detrimental, as anti-
CD40-mediated T-cell depletion may override the pro-survival effects of WBI. These questions remain to be evaluated.

In clinical trials, T cells expanded *ex vivo* to provide large doses for reinfusion into cancer patients are characterized by terminal differentiation and reduced potential to persist and self-renew (1). Recently the potential for dedifferentiation of patient-derived T cells from a terminal-effector toward a naïve- or stem-like phenotype has been highlighted (55). The addition of anti-CD40 to WBI conditioning could potentially reduce the demand for high numbers of expanded T cells, resulting in transfer of less-differentiated T cells that are desirable for use in ACT. Collectively, our results indicate that anti-CD40 conditioning can promote strong anti-tumor effects by ACT and raise the possibility of utilizing this approach alone or in combination with other immune interventions to achieve significant clinical benefits.

**References**


45. Wells JW, Cowled CJ, Farzaneh F, Noble A. Combined triggering of dendritic cell receptors results in synergistic activation and potent cytotoxic immunity. The Journal of


Figures and figure legends

Figure 4-1 Host conditioning with anti-CD40 induces high-level T cell accumulation in the lymphoid organs and brains of SV11 mice at early time points. Groups of mice received either anti-CD40, WBI, or no conditioning regimen with TCR-IV T cell ACT. *a,d*, Representative plots show MHC tetramer staining (mean±SEM) of TCR-IV T cells on days +4 and +5 in (a) spleen and (d) brain. *b,e*, Quantification of TCR-IV T-cell accumulation (mean±SEM) in (b) spleen and (e) brain of mice that received ACT with the indicated treatments. *c,f*, Total (c) splenocyte and (f) Percoll-enriched brain cell counts are shown (mean±SEM). n=3 mice/group (except n=2 for day +4 control group). Data shown are from one experiment and representative of two independent experiments. Asterisks above connecting lines indicate significant differences between time points. Asterisks next to vertical brackets indicate significant differences between treatment groups. *p < 0.05; **p < 0.01; ns: not significant
Figure 4-1
Figure 4-2 Anti-CD40-enhanced ACT promotes initial regression of established tumors. a, H&E brain sections on day +10 post-ACT following conditioning with anti-CD40 (left), control IgG (middle), or WBI (right). Representative low-power images (top row, scale bar = 1mm) and high-power images (bottom row, scale bar = 50µm) that show established tumor refractory to therapy (middle column) or tumor stromal condensation indicative of tumor regression (left and right columns). b, For each mouse, the largest cross-sectional tumor area (mm²) observed in H&E sections was plotted. Data is pooled from multiple experiments with a total of 6-10 mice/group. Statistical significance was determined using the Kruskal-Wallis test with Dunn’s multiple comparison test. **p < 0.01; ns: not significant
Figure 4-2

(a) Anti-CD40+ACT, Control IgG+ACT, WBI+ACT

(b) Tumor area (mm$^2$)

**ns**

**

Figure 4-2
Figure 4-3 Donor T cells contract dramatically in anti-CD40-conditioned SV11 mice. Groups of SV11 mice were treated as in figure 3-2 and sacrificed on days +6 and +10 post-ACT. a-d. Accumulation of tetramer-IV⁺ T cells is shown as percentage of CD8⁺ fraction in (a) spleen and (b) brain and as total number of TCR-IV T cells in (c) spleen and (d) brain. e-f. Using sample means in parts (c) and (d), the magnitude of TCR-IV T-cell contraction from day +6 to +10 was graphed for (e) spleen and (f) brain. Data are pooled from two experiments with a total of 3-6 mice/group. *p < 0.05; ***p < 0.001; ****p < 0.0001
Figure 4-3

(a) Spleen

(b) Brain

(c) Tetramer-IV T cells

(d) Tetramer-IV T cells

(e) Fold contraction

(f) Fold contraction
Figure 4-4. Phenotype of donor T cells at early time points. (a) Histograms illustrate expression of CD44 and CD62L on CD45.2^CD8^tetramer-IV^* T cells from spleen and brain on days +6 and +10 of representative mice that received either anti-CD40- (dashed blue line), control IgG- (filled histogram), or WBI-conditioning (solid red line) plus TCR-IV ACT. (b) Bar graphs indicate percent of live CD45.2^CD8^tetramer-IV^* T cells expressing KLRG1 (mean±SEM) in spleen and brain on (b) day +6 and (c) day +10. Results in part (b) are from a single experiment and results in part (c) are pooled from two independent experiments with a total of 3-7 mice/group. Samples with <25 tetramer-IV^* T cells were excluded. Statistical significance determined using one-way ANOVA with Bonferroni post-test. *p < 0.05; **p < 0.01
Figure 4-4

a

Day +6

Day +10

b

Spleen

Brain

Day +6

% KLRG1

Anti-CD40+ACT
Control IgG+ACT
WBI+ACT

Anti-CD40+ACT
Control IgG+ACT
WBI+ACT

Day +10

% KLRG1

Anti-CD40+ACT
Control IgG+ACT
WBI+ACT

Anti-CD40+ACT
Control IgG+ACT
WBI+ACT
Figure 4-5 Anti-CD40-enhanced ACT promotes increased survival but short-term surveillance against tumor recurrence. 

*a*, Groups of mice received the indicated conditioning with or without ACT and were monitored for tumor recurrence. The percentage of surviving mice versus age is plotted. 

*b*, Statistical differences in survival were calculated using the log-rank test. Data are pooled from multiple experiments with 5-8 mice/group. 

*c*, Representative H&E stained brain sections on day +135 post-ACT from WBI-conditioned mice in part (a) are shown. Low-power images (inset, scale bar = 1mm) show relative size and high-power images (scale bar = 100µm) show detailed histopathology of residual lesions. 

*d*, Tetramer-IV⁺ cells were detected in spleen and cLN of individual WBI-conditioned mice on day +135 post-ACT. Parallel results of site IV-specific intracellular cytokine staining and degranulation are plotted.
Figure 4-5

### a

![Graph showing percent survival over days of age for different groups.

### b

**Group:**
- Whole body irr. + TCR-IV: Median (days) >215, p value <0.001
- Anti-CD40 + TCR-IV: Median (days) 176
- Anti-CD40 + TCR-IV: Median (days) 176, p value 0.0043
- Control IgG + TCR-IV: Median (days) 136.5
- Anti-CD40 only: Median (days) 106, p value 0.5928
- Control IgG only: Median (days) 112
- Whole body irr. + TCR-IV: Median (days) >215, p value <0.001
- Whole body irr. only: Median (days) 119

### c

- **No tumor**
  - Representative of 6/7 mice
- **Tumor recurrence**
  - Representative of 1/7 mice

### d

- **Spleen**
- **cLN**

Table of median days and p values for different groups.

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Representative of 6/7 mice
Representative of 1/7 mice

Figure 4-5
Figure 4-6 Donor T cells fail to persist in anti-CD40-conditioned SV11 mice following acute tumor regression. Groups of SV11 mice received either anti-CD40, control IgG, or WBI conditioning prior to ACT with CD90.1⁺ TCR-IV T cells. a-b, On day +30, cells from (a) spleens, cLN (not shown), and (b) brains were stained for CD90.1 and CD8. Values on dot plots indicate percent CD90.1⁺ of total CD8⁺ cells (mean±SEM). c, Total CD90.1⁺ cells in spleens, cLN, and brains on day +30 are plotted. d-e, Representative histograms of CD44, CD62L, and KLRG1 expression gated on live CD45.2⁺CD8⁻CD90.1⁺ TCR-IV T cells (open histogram) or CD45.2⁺CD8⁻CD90.1⁻ T cells (filled histogram, spleen only) are shown on day +30 in (d) spleen and (e) brain. Values indicate the percent of TCR-IV T cells within the indicated gate (mean±SEM). Samples with <25 tetramer-IV⁺ T cells were not included in phenotype analyses. Data are representative of 3-5 mice/group. Statistical significance was determined using one-way ANOVA with Bonferroni post-test. **p < 0.01; ***p < 0.001; ****p < 0.0001; ns: not significant
Figure 4-6
Figure 4-7 Donor T cells do not emigrate to kidney, thymus, or bone marrow following acute tumor regression. Groups of SV11 mice received either anti-CD40 or WBI conditioning or no conditioning prior to ACT with CD90.1+ TCR-IV T cells. On day +20, cells from spleen, cLN, brains, kidney, thymus, and bone marrow were stained for CD90.1 and CD8. a, Representative dot plots are shown. Values on dot plots indicate percent CD90.1+ of total CD45.2+ cells. One untreated transgene-negative littermate (B6) was included as a negative staining control. b, The percent CD90.1+ of total CD45.2+ cells is graphed for each mouse. n=2-4 mice/group. Statistical significance was determined using one-way ANOVA with Bonferroni post-test. *p<0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns: not significant
Figure 4-7
Figure 4-8 TCR-IV T cells programmed in anti-CD40-conditioned mice are rescued following transfer into WBI-conditioned mice. a, Schematic of experimental design. Anti-CD40-conditioned SV11 mice received ACT with CD90.1⁺ TCR-IV T cells. On day +4, 1x10⁶ CD90.1⁺ splenocytes were sorted and transferred into WBI-conditioned SV11 and transgene negative (SV11⁻) mice. b, Surface staining phenotype of day +4 sorted CD90.1⁺ splenocytes. c, Quantification of CD90.1⁺ T-cell engraftment in spleen on day +5. d, Quantification of day +22 persisting CD90.1⁺ TCR-IV T cells in spleen, cLN, and brain of individual mice. e, KLRG-1 and CD127 staining on persisting CD90.1⁺ TCR-IV T cells on day +22 in spleen and brain of two SV11⁺ mice. Data are representative of 3-5 mice/group. *p < 0.05; ns: not significant
Figure 4-8
Figure 4-9 TCR-IV T cells programmed in anti-CD40- or WBI-conditioned mice possess similar survival and functional potential following transfer into WBI-conditioned control recipients. a, Schematic of experimental design. Anti-CD40- and WBI-conditioned SV11 mice received ACT with CD90.1+ TCR-IV T cells. On day +5, 1x10^6 CD90.1+ splenocytes were sorted and transferred into WBI-conditioned transgene negative (SV11') mice that had been immunized with a T Ag-expressing cell line on day +1. b, Surface staining phenotype of day +5 sorted CD90.1+ splenocytes. c, On day +25, cells from spleens were stained for CD45.2, CD8, and tetramer-IV. Values on dot plots indicate percent tetramer-IV+ of total CD45.2+ cells. d, Results of site IV-specific intracellular cytokine staining on day +25 are shown. n=3 mice/group. ns: not significant
Day: -1
SV11
Anti-CD40 or WBI
CD90.1+TCR-IV
Anti-CD40 or WBI
CD90.1+TCR-IV
Harvest and sort
Day: -1
SV11
WBI
T Ag immunization
ACT of sorted
CD90.1+TCR-IV
Analysis

Sorted from SV11 treated with:
anti-CD40+TCR-IV
WBI+TCR-IV

B6 recipients of:
Anti-CD40+TCR-IV donor cells
0.84
2.54
8.48
Tetramer-IV

WBI+TCR-IV donor cells
1.04
1.60
5.95

% cytokine+ of CD90.1 cells

Figure 4-9
Figure 4-10 Sustained peripheral depletion of CD8$^+$ T cells following WBI-enhanced ACT does not promote tumor recurrence or eliminate donor T cells from the brain. WBI-conditioned mice received ACT with TCR-IV T cells. Beginning on day +20, groups of mice received weekly injections of control antibody or depleting anti-CD8. a, Representative plots gated on TCRβ$^+$ cells from blood of control- and CD8-depleted mice stained for CD4 and CD8β on day +74. b, The fraction of CD8β$^+$ T cells in peripheral blood is plotted as % of TCRβ$^+$ cells on day +74. c, Quantification of tetramer-IV$^+$ T cells on day +80 in spleen and cLN. d, The cross-sectional area of the largest lesion in each brain was calculated and plotted. n=6 mice/group. e, Representative H&E brain sections show small residual lesions on day +80 in control- (left) and CD8-depleted (right) groups. Low-power images (scale bar = 1mm) show absence of gross lesions in rostral (upper) and caudal (lower) brain sections. High-power images (scale bar = 100µm) show residual microscopic anaplastic foci within the choroid plexus. f, Immunohistochemical staining for SV40 T antigen on brain sections from mice in part (d). g, In a second experiment using CD90.1$^+$ TCR-IV T cells, mice were harvested at day +80 post-ACT and CD90.1$^+$ T cells were quantified in all three tissues. n=4 mice/group. **p < 0.01; ***p < 0.001; ****p <0.0001; ns: not significant
**Figure 4-10** (continued next page)
Figure 4-10
Chapter 5

Discussion of major findings and their implications

Discussion

Host-conditioning regimens that support donor T cell survival and function improve the success of adoptive T cell transfer (ACT) therapy of cancer. Currently, chemotherapy and whole-body irradiation (WBI) are used as host-conditioning regimens in clinical trials of ACT therapy. Here, we sought to define the mechanisms of WBI host conditioning required for successful ACT-mediated regression of established tumors and protection from tumor recurrence. Initially, we hypothesized that the local effects of irradiation at the tumor site were required to promote ACT-mediated acute tumor regression. While our data suggest that the local effects of irradiation do promote ACT therapy, the most important effects of WBI host conditioning are systemic, not local. We investigated the impact of host conditioning on the donor T cell response at early and late time points following ACT and found that host conditioning has significant influence on donor T cell accumulation, tumor regression, donor T cell persistence, and long-term survival. This study also reveals that agonist anti-CD40 can substitute for WBI conditioning and effectively promote acute tumor regression in the absence of irradiation, but that long-term protection from tumor recurrence requires the systemic effects of irradiation conditioning.
The local effects of WBI conditioning at the tumor site are not required to promote ACT-mediated tumor regression and long-term survival.

Our studies on the impact of the timing of ACT revealed that WBI conditioning induces a prolonged window of opportunity during which ACT therapy can successfully promote acute tumor regression, but that when ACT is delayed after WBI, the long-term survival benefit is compromised. This finding was unexpected, as complete tumor regression and donor T cell accumulation were found to be equivalent at multiple time points following early and delayed ACT. We reasoned that the host environment at the time of ACT plays a critical role in determining the long-term therapeutic outcome. In particular, WBI conditioning-induced systemic changes and local changes to the tumor microenvironment (TME) are likely to effect response to ACT therapy.

We found that the local effects of irradiation on the TME are not required to promote ACT-mediated tumor regression or long-term protection from recurrence. Previous studies of ACT therapy by other research groups have demonstrated that the requirement for local irradiation of the tumor site varies and may be tumor model-specific (1,2). Local irradiation can promote the activation of tumor-infiltrating APCs and their subsequent migration to draining lymph nodes. This migration may result in donor T cell activation if these irradiation-activated APCs have taken-up and presented tumor antigens. We have demonstrated enhanced donor T cell accumulation in the tumor-draining lymph nodes following local irradiation, suggesting this mechanism is operational during ACT therapy of SV11 mice. Our results using irradiation focused to the body suggest that this mechanism is not required to promote successful ACT therapy in SV11 mice. However, it is important to note that SV11 mice express low-levels of T antigen in the kidney. Therefore, irradiation of the body, even in the absence of local irradiation to the tumor, may activate kidney-resident APCs to present T antigen and activate donor T cells. Therefore, local irradiation of the tumor site may be required to enhance ACT therapy by
promoting tumor antigen-presentation in situations in which tumor antigen expression is restricted solely to the tumor site.

As opposed to the local effects of WBI, the systemic effects of WBI conditioning at the time of ACT are a crucial determinant of ACT therapeutic success. Because WBI-induced lymphodepletion transiently increases the availability of T cell survival cytokines, ACT early after WBI would be expected to result in higher levels of donor T cell accumulation. In support of this notion, we found that the administration of ACT early after WBI resulted in enhanced donor T cell enrichment early after ACT. Further analysis is required to determine whether this enrichment correlates with an increase in total donor T cell number, as differences in the rate of recovery of other immune cell populations post-WBI may contribute to the relative enrichment of donor T cells without increase in absolute number. These findings define a short-lived “critical period” following WBI conditioning that is the optimal time to administer ACT (Fig. 5-1). The model illustrated in figure 5-1 depicts the transient increase in survival cytokine availability following lymphodepleting WBI conditioning. As the host recovers from lymphopenia, survival cytokine availability returns to baseline. ACT administered early after WBI results in rapid donor T cell accumulation. Conversely, delay of ACT after WBI misses the critical period, and donor T cell accumulation proceeds at a slower rate. The homeostatic T cell cytokines IL-7 and IL-15 are most likely the survival cytokines that support donor T cell accumulation early after WBI, however, experiments that specifically deplete these cytokines individually and collectively following WBI conditioning will be required to define the factors critical for optimal donor T cell accumulation.

Alternatively, enhanced donor T cell accumulation following early ACT may not be mediated by increased cytokine availability. Instead, donor T cells administered early after WBI may benefit from a brief reprieve from homeostatic suppressive mechanisms that are progressively restored in the days following WBI conditioning. TGF-β is an immunosuppressive
cytokine produced by \( T_{\text{REG}} \) cells that can suppress the proliferation of activated T cells. Memory T cell homeostasis is maintained by a balance of IL-15 and TGF-\( \beta \) (3). Following WBI-induced lymphodepletion, the increased levels of available IL-15 may induce a counter-regulatory up-regulation of TGF-\( \beta \) production by \( T_{\text{REG}} \) cells. Importantly, an enrichment in \( T_{\text{REG}} \) cells has been demonstrated in mice due to brisk repopulation following the administration of 5 Gy WBI (4). Therefore, it is possible that by the time donor T cells begin to proliferate following delayed ACT, both \( T_{\text{REG}} \) cells and TGF-\( \beta \) levels have recovered to mediate active suppression of donor T cell proliferation. An analogous effect has been demonstrated in a mouse strain that overexpresses TGF-\( \beta \) (5). Following 5 Gy WBI, these TGF-\( \beta \)-overexpressing mice are impaired in their ability to repopulate the hematopoietic compartment compared to wild-type mice. Whether mediated by an increase in cytokine support or a decrease in T cell-suppression, the critical period that immediately follows WBI conditioning is an important window of time that must be considered during design and practice of ACT therapy. It is important to note that the kinetics of lymphodepletion and cytokine availability may vary based on the host-conditioning regimen used. Ideally, a short-acting lymphodepleting agent could be administered immediately before ACT to maximize the benefits of lymphodepletion on the accumulating donor T cells.

Additionally, the phenotype and activation state of donor T cells at the time of ACT may dictate the optimal timing of ACT, as highly-activated T cells, which express higher levels of cytokine receptors, may require immediate cytokine availability and therefore be optimally transferred after a delay, when survival cytokine availability is at peak levels.

We have demonstrated that differences in the early accumulation of T cells following ACT can manifest as significant reductions in overall survival at much later time points. This will be an especially important consideration as the field of ACT therapy continues to progress, and long-term regressions and cures become the standard. These findings imply that rapid, high-level T cell accumulation at the tumor site should be a primary goal, in addition to tumor regression,
because this provides the best chance of establishing persistent anti-tumor T cells at the tumor site and preventing recurrence. While not demonstrated here, others have shown that tumors up-regulate resistance mechanisms in responses to T cell infiltration, such as the expression of the inhibitory ligand PD-L1 by tumor cells following infiltration by IFNγ-expressing effector T cells (6). Rapid accumulation of T cells at the tumor site may represent the best approach to outpace the up-regulation of these tumor-resistance mechanisms.

Additionally, we have demonstrated that local irradiation of the tumor site is not required to promote ACT-mediated tumor regression or long-term survival. This finding has direct implications on modern ACT therapy, which uses WBI as part of the host-conditioning regimen. Further refinements to this protocol should include a reduction in the extent of the irradiation field to exclude all non-essential tissues from unnecessary exposure. In light of our finding that systemic lymphodepletion is a critical determinant of successful ACT therapy, we hypothesize that local irradiation to select lymphoid tissue is adequate to induce sufficient lymphodepletion and promote successful ACT therapy. Future research should investigate the use of local irradiation focused to specific lymphoid tissues, in particular the spleen and tumor-draining lymph nodes, to induce lymphodepletion without affecting other tissues. Furthermore, this focused lymphodepletion may avoid the susceptibility to opportunistic infections associated with transient immune-depletion.

Rapid, high-level donor T cell accumulation at the tumor site is sufficient to promote ACT-mediated regression of established tumors but not long-term protection from tumor recurrence

Given our findings that the local effects of irradiation at the tumor site are not required for ACT-mediated tumor regression or to promote donor T cell persistence at the tumor site, and that the magnitude of donor T cell accumulation early after ACT is the predominant factor
predicting a successful response to therapy, then it stands to reason that alternative conditioning approaches that promote high-level donor T cell accumulation should also promote ACT-mediated tumor regression and long-term protection. By directly comparing anti-CD40 and WBI conditioning, we found that anti-CD40 conditioning was indeed sufficient to promote ACT-mediated tumor regression in the absence of irradiation. However, anti-CD40 was unable to recapitulate the extended survival observed using WBI conditioning.

In contrast to our previous findings with delayed ACT, there was no impairment in early donor T cell accumulation in anti-CD40-conditioned mice. However, early tumor recurrence in anti-CD40-conditioned mice was associated with a nearly complete contraction of the donor T cell populations in the lymphoid organs and in the brain shortly after tumor regression. Given that donor T cells accumulate and persist long-term in WBI-conditioned mice, the loss of persistence in anti-CD40-conditioned mice suggests that a block in donor T cell differentiation is preventing the formation of a persistent memory-like T cell population (Fig. 5-2a and b). We investigated possible explanations for this block in differentiation, and found that donor T cells isolated from anti-CD40-conditioned mice early after ACT can be rescued by transfer into a WBI-conditioned recipient, and that this rescue is dependent on the presence of cognate antigen in the recipient. This finding illustrates three important qualities of the block in T cell differentiation toward a persisting memory-like T cell population. First, this finding demonstrates that the initial activation by anti-CD40-licensed APCs does not irreversibly determine the fate of donor T cells. Second, this finding demonstrates that these rescued donor T cells are receiving a critical survival signal in the lymphopenic host that was absent or unavailable in the anti-CD40-conditioned host. Third, this finding demonstrates that survival of these rescued T cells requires interaction with an APC presenting cognate antigen. Collectively, these qualities suggest that the block in differentiation most likely occurs after initial T cell activation and before migration to the tumor. Tumor-induced suppression of donor T cell differentiation is highly unlikely considering the
majority of donor T cells in anti-CD40-conditioned mice do not accumulate at the tumor site, and yet these cells also contract. Thus, donor T cell accumulation following activation by anti-CD40-licensed APCs may be occurring in the absence of an important survival signal. Furthermore, this unknown survival signal is present in WBI-conditioned mice and is mediated by APCs. IL-15 is an important survival cytokine for activated and memory T cells. It is predominantly produced by APCs and presented to T cells at the APC membrane bound to the high-affinity IL-15Rα chain (7). Furthermore, IL-15 has previously been demonstrated to be a limiting factor in other models of anti-CD40 immunotherapy (8). Collectively, this suggests that the administration of exogenous IL-15 with anti-CD40 therapy may promote the differentiation of donor T cells toward long-lived persistent memory-like T cells, resulting in long-term protection (Fig. 5-2c).

Here, we have demonstrated that the rapid accumulation of donor T cells at the tumor site is sufficient to promote ACT-mediated regression of established tumors, but that donor T cells contract rapidly following tumor regression, likely due to the absence of additional stimuli to promote T cell persistence. These findings have implications for the use of anti-CD40 in combination with ACT therapy, which is yet to be investigated in human patients. We hypothesize that the combination of anti-CD40 with additional immune modulating agents that promote donor T cell persistence at the tumor site will be sufficient to promote long-term survival and protection from tumor recurrence. Future directions in this project should further investigate the mechanisms of anti-CD40-mediated T cell contraction, as this has direct implications for current practice. In particular, investigation of the mechanisms required to promote donor T cell persistence at the tumor site following tumor regression will provide valuable insight into the shortcomings of many T cell immunotherapies, including ACT therapy, in which complete responses can be achieved only to recur months or years later. Previous studies from our group investigated the combination of anti-CD40+ACT in the SV11 model using donor T cells targeting the immunorecessive site V determinant. While this initial approach was unsuccessful, the
inclusion of a well-timed vaccination with a T antigen-expressing cell line resulted in enhanced donor T cell accumulation in the brain and donor T cell persistence beyond 40 days post-ACT (9). Consideration of these results with the current study suggests that the addition of tumor antigen-vaccination to anti-CD40+ACT therapy using TCR-IV donor T cells may be sufficient to promote donor T cell persistence and long-term survival.

A major advantage to anti-CD40 therapy is that it enhances priming against the endogenous tumor antigens and can therefore promote a polyclonal anti-tumor response. Neoantigens derived from mutated gene products represent an important target for T cell immunotherapies. These tumor-specific antigens can be targeted by high-affinity T cells that are not deleted by thymic selection. The ability to prime or amplify T cell responses against unique neoantigens in vivo represents an important mechanism that can be exploited by anti-CD40.

Extensive analysis of tumor-infiltrating T cell clones in pre- and post-treatment surgical specimens revealed a substantial broadening of the tumor-infiltrating T cell repertoire in a melanoma patient that eventually experienced a complete response to anti-CD40 therapy (10). Thus, anti-CD40 therapy could even be used to diversify the endogenous TIL repertoire before tumor resection and TIL ex vivo expansion for ACT therapy. In addition to broadening the TIL repertoire, periodic anti-CD40 administration could be used to maintain long-term immunological tumor control by promoting the activation of new endogenous T cell clones that recognize tumor neoantigens that arise over time, in effect, protecting against the outgrowth of antigen-loss variant tumors. Because antigen loss has not been observed in SV11 mice, future testing of this hypothesis will require use of an alternative mouse model that is susceptible to cancer immunoediting and antigen loss by tumor cells, such as a transplantable tumor model or a carcinogen-induced tumor model. In summary, the combination of anti-CD40 with ACT therapy shows promise for promoting in vivo expansion and accumulation of donor T cells as well as the induction of endogenous T cell responses against novel tumor antigens that arise over time.
**Donor T cell persistence at the tumor site is associated with long-term tumor control**

Perhaps the most interesting finding in the current study is the identification of persisting donor T cells in the brains of WBI-conditioned mice following tumor regression that were resistant to antibody-mediated depletion. This resistance suggests these brain-resident T cells are a non-circulating population of anti-tumor T cells that may protect against tumor recurrence. Evidence from mouse models of viral infection have identified a resident memory T cell (T\text{RM}) subset that is retained in the tissue at the site of previous infection, including in the brain (11), where they can provide first-line protection against subsequent infection. T\text{RM} cells established in the brain up-regulate expression of the integrin CD103, which binds E-cadherin expressed on epithelial cells and may be required to retain these cells in tissue. Interestingly, we have observed that a significant fraction of persisting TCR-IV T cells in the brain following WBI+ACT therapy are CD103\textsuperscript{+} (unpublished observations), suggesting these persistent donor T cells may represent \textit{bona fide} anti-tumor T\text{RM} cells. To our knowledge, this is the first demonstration of long-lived, non-circulating brain-resident anti-tumor T cells established following tumor regression. Future studies utilizing alternative depletion methods that allow donor T cell depletion from the brain will be necessary to determine whether these persisting donor T cells are absolutely required for long-term protection against tumor recurrence.

The kinetics and mechanisms of the establishment of these persistent donor T cells following tumor regression have not been defined. Donor T cells persist in the brains of mice conditioned with irradiation focused to the body, suggesting that local modulation of the TME is not required to establish persisting donor T cells. Instead, early donor T cell accumulation and differentiation may prove to be an important factor in generating these cells. We did not compare the magnitude of persisting donor T cells in the brains of mice that received either early or delayed ACT, however, we hypothesize that early ACT promotes enhanced accumulation of
persisting donor T cells in the brain secondary to enhanced accumulation early after ACT. Conversely, a reduction in early donor T cell accumulation will compromise the establishment of persisting donor T cells and long-term survival.

While the magnitude of early donor T cell accumulation may be an important determinant of the establishment of persisting donor T cells, other factors also likely influence this mechanism. Anti-CD40 conditioning promotes early donor T cell accumulation in the brain to a similar magnitude as WBI conditioning, yet persisting donor T cells are only established following the latter treatment. As discussed earlier, the absence of pro-survival factors in the anti-CD40-conditioned host may preclude the differentiation of persisting donor T cells in the lymphoid organs and at the tumor site. Future studies utilizing antibody-based T cell depletion can define whether long-lived donor T cells persisting in the brain are derived from early- or late-accumulating donor T cells and may inform the design of new therapeutic protocols to promote donor T cell persistence, in particular the timely administration of exogenous survival cytokines or other pro-survival factors.

Future experimental studies will also be interesting to determine whether these persisting donor T cells can be isolated from the tumor site and adoptively transferred to provide long-term protection against tumor recurrence to recipients. Previous studies in viral infection models have shown that T_{RM} cells die rapidly in culture; however, if persisting donor T cells in the brain can be isolated, cultured, and used therapeutically, then it may be possible to differentiate donor T cells to T_{RM}-like cells in culture prior to ACT. An understanding of the mechanisms of T_{RM} differentiation and establishment can be translated to clinical practice immediately. Currently, little is known about the existence or function of anti-tumor memory-like T cells that may persist at the tumor site long after tumor regression in human patients. Clinical studies to determine the existence and phenotype of these cells following immunotherapy-mediated tumor regressions represent an important step to characterize this potentially critical anti-tumor immune cell
population. For example, skin biopsies from melanoma patients that achieved complete responses following ACT or other immunotherapy can be utilized to attempt to identify these “\(T_{RM}\)-like” cells by microscopy or in vitro isolation and culture. Following this characterization, clinical trials could be designed to optimize host-conditioning regimens for their ability to promote the establishment of “\(T_{RM}\)-like” anti-tumor T cells and determine the impact of this optimization on long-term protection from tumor recurrence.

Conclusions and future outlook

In summary, we have demonstrated that the systemic effects of WBI conditioning are critical to promote successful ACT therapy. Furthermore, WBI conditioning need not include the tumor site in the irradiation field, suggesting targeted irradiation of select lymphoid tissues may be sufficient to promote successful ACT therapy while also reducing overall exposure to irradiation, which can cause severe and long-lasting side effects. Following successful ACT-mediated tumor regression, persistent donor T cells can be established at the tumor site and may represent a critical immune population to provide long-term protection against tumor recurrence. Finally, anti-CD40 is a promising agent that can promote ACT-mediated tumor regression but requires further refinement to achieve a long-lasting response.

Collectively, these findings imply that the mechanisms for ACT-mediated tumor regression and protection from recurrence are distinct, and that multiple conditioning approaches may be required to replicate the effects of WBI. The application of ACT therapy to early stage cancers may provide an impetus for the disuse of irradiation conditioning altogether, as the risk of potential side effects may be greater than patients with less aggressive tumors are willing to accept. Successful treatment of more refractory tumor types, on the other hand, may require more aggressive host-conditioning strategies, such as the combination of WBI and anti-CD40.
A recent study has highlighted the possibility of foregoing the use of host-conditioning regimens in ACT therapy by enhancing the intrinsic abilities of donor T cells to persist and execute effector functions (12). Yun et al. demonstrated that overexpression of the microRNA miR-155 in donor T cells was sufficient to promote successful ACT therapy of murine melanoma. Importantly, combination with WBI conditioning or exogenous cytokine administration did not further enhance this therapy, indicating that miR-155 overexpression can effectively substitute for multiple host-conditioning approaches, making this approach potentially applicable to a larger patient population for whom current host-conditioning regimens are contraindicated, such as those with cardiovascular issues. This result suggests that future development of ACT therapy may lie in approaches that overcome the need for aggressive host-conditioning regimens by improving the donor T cell-intrinsic functions prior to infusion into the cancer patient.

Overall, our findings suggest that the optimal cancer immunotherapy incorporates in vivo activation of tumor-specific T cells against endogenous tumor antigens, rapid accumulation of activated T cells at the tumor site to induce tumor regression, and long-term persistence of anti-tumor T cells at the tumor site to prevent recurrence. At present, irradiation conditioning remains the best method to achieve these goals. To advance beyond the use of irradiation and its associated side effects, novel conditioning strategies must be developed that stimulate the initial anti-tumor response to induce tumor regression and promote the establishment of long-lasting immunosurveillance.

References


Figure 5-1 The administration of ACT during a critical period of maximum survival cytokine availability is required to optimize therapeutic benefit. WBI conditioning induces systemic lymphodepletion and transiently increases the availability of T cell survival cytokines. a, Delay of ACT after WBI conditioning misses the critical period, resulting in impaired donor T cell accumulation. b, ACT early after WBI conditioning results in rapid donor T cell accumulation due to increased availability of survival cytokines during the critical period.
Figure 5-1

**a** Delayed ACT after WBI

**b** ACT early after WBI

- **Total lymphocytes**
- **Donor T cells**
- **Survival cytokine availability**
- **WBI**
- **Critical period**
Figure 5-2 Model of WBI conditioning and anti-CD40 conditioning for ACT therapy. 

a, Following activation in a WBI-conditioned host, donor T cells accumulate and differentiate into short-lived effectors and long-lived persistent T cells in the presence of host APCs bearing endogenous IL-15, migrate to the tumor site, mediate tumor regression, and persist at the tumor site to protect from recurrence. 

b, Following activation in an anti-CD40-conditioned host, donor T cells accumulate independent of host APCs, migrate to the tumor site, mediate tumor regression, and do not persist, resulting in tumor recurrence. 

c, Following activation in an anti-CD40-conditioned host, donor T cells accumulate and differentiate into short-lived effectors and long-lived persistent T cells in the presence of host APCs bearing exogenous IL-15, migrate to the tumor site, mediate tumor regression, and persist at the tumor site to protect from recurrence.
Figure 5-2
VITA

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