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TOWARD DESIGNING A MORE EFFECTIVE VACCINE:

MECHANISMS OF PRIMING T CELLS *IN VIVO*

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Integrative Biosciences

by

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ABSTRACT

The infectious diseases that currently pose the greatest threat to humans, AIDS, tuberculosis and malaria, are likely to require a vaccine strategy designed to elicit T cell-mediated immunity. Rational vaccine design requires a mechanistic understanding of how T cells are activated *in vivo*. Thus, we sought to examine the requirements of the direct and cross-priming pathways of CD8+ T cell activation as well as examine the antigen processing and presentation pathways available to a model antigen when expressed by viral vaccine vectors. Investigation of the requirements for direct and cross-priming revealed that the two pathways utilize different pools of antigen. Direct presentation requires newly synthesized, rapidly degraded antigen while cross-priming requires long-lived stable antigen. Additionally, antigen donor cells had no requirement for proteasomal processing, protein synthesis or vesicular transport of the antigen or other cellular factors in order to donate antigen to the cross-priming pathway *in vivo*. Investigation of the antigen processing and presentation pathways available to a model antigen, OVA, when expressed from viral vectors produced unexpected results. We found that recombinant Vaccinia Virus (rVV) expressed OVA was permitted to access the TAP-independent cross-priming pathway- a pathway which OVA is normally unable to access. Although rVV expression permitted the model antigen to access an additional antigen processing and presentation pathway, recombinant Vesicular Stomatitis Virus (rVSV) expression stimulated 5-fold more model antigen-specific CD8+ T cells. Initially, rVSV-expressed OVA also appeared to require TAP for MHC class II presentation. However, subsequent experiments revealed that the lack of presentation in TAP knockout mice was not due to TAP-dependent MHC class II presentation of rVSV-expressed OVA but instead due to a lack of antigen presenting cell subsets in the gamma-irradiated TAP knockout mice. Together these findings have broad implications for the rational design of vaccines aimed at the generation of protective T cell responses.

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LIST OF ABBREVIATIONS

β gal	β-galactosidase
β_2m	β_2-microglobulin
$\beta_2$mneg	β_2-microglobulin negative
1E12	Murine TAP-/- fibroblasts
293 A	Human embryonic kidney cells
APC	Antigen Presenting Cells
B3Z	CD8+ T cell hybridoma specific for $OVA_{257-264}$ complexed to K^b that produces β gal upon activation
B6	C57BL/6 mice
BFA	Brefeldin A
Bg1	TCR transgenic mice that express CD8+ T cells specific for β gal_{96-103} complexed to H2-K^b
Bg1.SJL	Bg1 mice crossed with B6.SJL-Ptprca/BoAiTac mice so that the transgenic T cells can be identified via the CD45.1 marker
BMDC	Bone Marrow Dendritic Cells
BO-80.10	$OVA_{323-339}$-specific hybridoma
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CLIP	Class II associated Invariant chain Peptide
DC	Dendritic Cell
DRiP	Defective Ribosomal Products
ER	Endoplasmic Reticulum

ERAAP	ER Aminopeptidase associated with Antigen Processing
Fc	Fragment crystallizable; refers to the constant domain of antibodies
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescent Protein
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HSP	Heat Shock Protein
IAV	Influenza A Virus
ICS	Intracellular Cytokine Staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneally
ISCOM	Immunostimulatory complexes
IV	Intravenously
L4.2	293 cells stably expressing the tetracycline repressor transfected with a plasmid for expressing β gal under the tetracycline operator
L4.2mK^b	L4.2 cells transduced with a retrovirus expressing murine MHC class I molecule H2-K^b
MHC	Major Histocompatibility Complex
NP	Nuclear Protein from IAV
OT2Z	CD4+ T cell hybridoma specific for $OVA_{323-339}$ complexed to I-A^b that produces β gal upon activation

OT-I	TCR transgenic mice expressing a CD8+ TCR specific for $OVA_{257-264}$ complexed to K^b
OT-I.SJL	OT-I mice crossed with B6.SJL-Ptprca/BoAiTac mice so that the transgenic T cells can be identified via the CD45.1 marker
OT-II	TCR transgenic mice expressing a CD4+ TCR specific for $OVA_{323-339}$ complexed to $I\text{-}A^b$
OT-II.SJL	OT-II mice crossed with B6.SJL-Ptprca/BoAiTac mice so that the transgenic T cells can be identified via the CD45.1 marker
OVA	Ovalbumin
$OVA_{257-264}$	Immunodominant MHC class I epitope of OVA; also called the SIINFEKL peptide
$OVA_{323-339}$	Immunodominant MHC class II epitope of OVA
P815	Murine mast cell expressing $H\text{-}2^d$
pAPC	Professional Antigen Presenting Cells
PFU	Plaque Forming Unit
rVSV	Recombinant Vesicular Stomatitis Virus
rVV	Recombinant Vaccinia Virus
SIINFEKL	Immunodominant MHC class I epitope of OVA; also called $OVA_{257-264}$
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TPPII	Tripeptidyl protease II
Ub	Ubiquitin

UV	Ultraviolet
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
WT3	Murine fibroblasts
X-gal	5-bromo-4-chloro-3-inodolyl-β-D galactopyranoside

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Chapter 1

Introduction

T cells play an essential role in protective immunity to viruses and tumors. In spite of this, little is known about how T cell immunity is stimulated *in vivo*. In order to efficiently activate T cells *in vivo* using vaccines, it is necessary to have a better understanding of the antigen processing and presentation pathways used to activate T cells *in vivo*. Thus, our experiments will examine the *in vivo* antigen processing and presentation pathways in two manners. First, we will examine the requirements of the MHC class I processing pathways. Then, we will examine the antigen processing pathways available to an antigen expressed by different viruses. To better frame the questions that will be examined in our experiments, it is necessary to review what is currently known about stimulating T cells. Consequently, T cells, professional antigen presenting cells, and the antigen processing and presentation pathways will be reviewed in this chapter. Additionally, the life cycles and immune responses stimulated by the two viral vaccine vectors that will be used in our experiments will also be reviewed.

1.1 T cells

T cells are the cellular component of the adaptive immune response. A central feature of the adaptive immune response is the ability to generate an incredibly flexible repertoire of receptors by rearrangement of receptor gene fragments (Davis and Bjorkman, 1988; Weigert et al., 1978). The diversity of the receptors expressed on the cell surface of T cells permits specific recognition of pathogens as well immunological memory, the ability to respond immediately and

specifically to subsequent challenges by the same pathogen (Vitetta et al., 1991). T cells are derived from bone marrow stem cells and differentiate in the thymus. The differentiation process results in the elimination of approximately 98% of the T cell precursors that enter the thymus (Shortman et al., 1990). T cells exiting the thymus are referred to as naïve T cells. Naïve T cells have fully functional T cell receptors, but are incapable of responding to antigen-MHC complexes in the absence of co-stimulation from a professional antigen presenting cell (pAPC) (Germain, 1981; Roska and Lipsky, 1985). T cell receptors recognize a peptide processed from a protein antigen and presented on MHC (Zinkernagel and Doherty, 1974). Simultaneously, a co-receptor, either CD4 or CD8, recognizes the MHC molecule itself. T cells expressing CD8 recognize MHC class I molecules while those expressing CD4 recognize MHC class II molecules. Recognition by both T cell receptor and co-receptor produces a signal which activates a T cell response. However, in the absence of an additional signal from a co-stimulatory molecule on the pAPC, a naïve T cell will be unable to respond; a method by which the immune system prevents self-reactive T cells. The best-characterized co-stimulatory molecules expressed on pAPC are the cell surface glycoproteins B7.1 (CD80) and B7.2 (CD86), which bind to CD28 molecules expressed on T cells. The binding of CD28 to B7.1 or B7.2 initiates the second signal needed for T cell activation (Bhatia et al., 2006; Lenschow et al., 1996). Upon receipt of the second signal, T cells secrete interleukin 2 (IL-2) and express the high affinity IL-2 receptor. IL-2 stimulates T cells to proliferate and differentiate into effector T cells. Our studies use the proliferation of T cells as a measure of T cell activation due to antigen presentation. Following less than 30 hours of rapid growth, T cells acquire effector function (Oehen and Brduscha-Riem, 1998). Subsequent recognition of MHC-antigen complexes by effector T cells results in immediate effector function without a requirement for a secondary signal, although recent experiments have demonstrated that secondary signals enhance memory effector T cell responses *in vivo* (Borowski et al., 2007; Fuse et al., 2008; Ndejembi et al., 2006). The effector function acquired depends on the type of T cell,

CD8+ or CD4+, as well as the cytokines present during activation of a naïve T cell. As the different effector functions are used to determine the activation status of T cells in our studies, it is important to understand the differences between CD8+ and CD4+ T cells.

1.1.1 CD8+ T cells

CD8+ T cells recognize antigen presented on MHC class I and play an important role in the removal of intracellular pathogens, infected cells, tumors and transplanted tissues. CD8+ T cells are also called cytotoxic T cells because one of their effector functions is to kill cells identified as infected by the presence of antigen on MHC class I (Ashton-Rickardt, 2005; Russell and Ley, 2002). CD8+ T cells also secrete cytokines that help mediate immunity. Interferon-γ (IFN-γ), tumor necrosis factors α and β(TNF-α and TNF-β) and lymphotoxin can all be secreted by CD8+ T cells. These inflammatory cytokines up-regulate cytotoxic functions of macrophages and granulocytes (Fong and Mosmann, 1990). Up-regulation of the cytotoxic functions of macrophages and granulocytes serves two purposes. One, it stimulates macrophages, a type of pAPC, to enhance the antigen processing and presentation pathways. Expression of the immunoproteasome subunits and up-regulated expression of the transporter associated with antigen processing (TAP) enhance the antigen processing pathways while up-regulated expression of MHC class I and II enhances antigen presentation (Chang et al., 1992a; Epperson et al., 1992; Figueiredo et al., 1989; Groettrup et al., 2001; Hisamatsu et al., 1996; Shirayoshi et al., 1988). Two, up-regulated cytotoxic function further activates granulocytes to release their granules; effectively recruiting innate immune cells to help clear a site of infection. Secretion of IFN-γ also functions to prevent infection of bystander cells when CD8+ T cells lyse virally infected cells. Several of our studies measure activation of CD8+ T cells by Intracellular Cytokine Staining (ICS) for IFN-γ.

1.1.2 CD4+ T cells

CD4+ T cells recognize antigen presented on MHC class II and play an important role in the "helper" responses of activating both antigen presenting cells and CD8+ T cells as well as enhancing activation of the humoral response. For this reason, CD4+ T cells are also referred to as helper T cells. The two subsets of helper T cells are differentiated by the cytokines that they secrete (Kamogawa et al., 1993). One subset primarily secretes IFN-γ while the other primarily secretes IL-4. However, both subsets secrete, IL-2. Thus, our studies used ICS for IL-2, IL-4 and IFN-γ to identify activated CD4+ T cells.

Another function of CD4+ T cells is that they are necessary for the establishment and maintenance of immunological memory. Early work showed that CD4+ T cell help was necessary for the establishment of a CD8+ T cell responses (Cassell and Forman, 1988; Husmann and Bevan, 1988; Keene and Forman, 1982). Subsequent studies then contradicted the requirement for CD4+ T cell help in the establishment of CD8+ T cell responses in acute viral infections (Buller et al., 1987; Rahemtulla et al., 1991; Wu and Liu, 1994). However, CD4+ T cell help was required for CD8+ T cell responses to non-inflammatory cellular antigens (Bourgeois et al., 2002; Fernando et al., 2002; Janssen et al., 2003) and antigens from chronic infection (Matloubian et al., 1994; Wang and Livingstone, 2003). Recent studies have demonstrated that CD4+ T cell help is required during the first few days of a CD8+ T cell response in order to generate functional CD8+ T cell memory (Fernando et al., 2002; Shedlock and Shen, 2003; Sun and Bevan, 2003). Mice lacking CD4+ T cells were able to generate normal primary CD8+ T cells responses but were unable to generate a memory CD8+ T cell response (Badovinac et al., 2002; van Stipdonk et al., 2003; van Stipdonk et al., 2001). Thus, the activation of CD4+ T cells as well as CD8+ T cells will likely be a requirement for vaccines designed to stimulate protective T cell immunity.

1.2 Professional Antigen Presenting Cells (pAPC)

While most cell types in the body constitutively express MHC class I molecules and are capable of stimulating an immune response from effector T cells, only pAPC are capable of providing the co-stimulatory signal necessary to activate naïve T cells. pAPC also express MHC class II and are able to stimulate a response from CD4+ T cells (Cowing et al., 1978; Yamashita and Shevach, 1977). The pAPC are dendritic cells (DC), macrophages, and B cells, although other cells can become pAPC (Brandes et al., 2005; Epperson and Pober, 1994). However, only DC, macrophages and B cells will be discussed in detail below.

DC are derived from bone marrow precursors and express a wide array of pathogen recognition receptors, complement receptors (which recognize the activated complement components of the innate immune system) and Fc receptors (which recognize the constant, or Fc portion of antibodies) that permit acquisition of antigen by receptor-mediated endocytosis (Taylor et al., 2005). DC found in the periphery (outside the lymphoid organs) are immature and continually sample their microenvironments through macropinocytosis (Norbury et al., 1997; Sallusto et al., 1995; West et al., 1999). Immature DC are poor stimulators of naïve T cells and may induce tolerance through a lack of co-stimulatory signal (Finkelman et al., 1996; Hawiger et al., 2001; Schuler and Steinman, 1985; Winzler et al., 1997). DC maturation is stimulated either through inflammatory mediators or through ligands binding to pattern recognition receptors present on the cell surface of DC. Maturation stimuli activate DC, permitting DC to migrate through the lymphatics to the secondary lymphoid tissues (Larsen et al., 1990). Maturation stimuli also transiently up-regulate the macropinocytosis and phagocytosis pathways in maturing DC (West et al., 2004). Upon maturation, DC lose the ability to take up antigens as antigen processing and presentation increase (Delamarre et al., 2003; Reis e Sousa et al., 1993; West et al., 2004). At this time, MHC class I and II expression on the cell surface increases, as does

expression of co-stimulatory molecules and adhesion molecules (Larsen et al., 1992). Once in the lymphoid tissues, DC begin to secrete chemokines to attract T cells, allowing presentation of antigen to and activation of naïve T cells.

Macrophages are derived from blood monocytes and are present in all tissues (Gordon, 1998). Like DC, macrophages express a wide array of pathogen recognition receptors, complement receptors and Fc receptors that permit them to acquire antigen by receptor-mediated endocytosis (Taylor et al., 2005). Macrophages function as phagocytic cells in the innate immune system by ingesting microbes to prevent the microbes from infecting other tissues. When these microbes persist or evade elimination by the innate immune system, macrophages can be activated to serve as antigen presenting cells as part of the adaptive immune response. Resting (non-activated) macrophages express few or no MHC class II molecules and no co-stimulatory molecules on their cell surface (Gordon and Taylor, 2005). Activation of macrophages by recognition of a pathogen on a pathogen recognition receptor, or by IFN-γ induces expression of MHC class II and co-stimulatory molecules such as B7 (Dalton et al., 1993; Lang, 2005) on the cell surface of macrophages, permitting activation of naïve T cells.

B cells are derived from bone marrow precursors and are adapted to internalize and present antigens from soluble molecules recognized by cell surface immunoglobulin receptors (Davidson et al., 1991). However, B cells can also take up pathogens via Fc receptor recognition of antibody-antigen complexes (Kakiuchi et al., 1983; Lanzavecchia, 1985; Lanzavecchia, 1986). MHC class I and II are constitutively expressed by B cells although MHC class II expression is up-regulated upon B cell activation; high levels of MHC class II-peptide complexes are found on activated B cells. Although B cells do take up and present soluble self antigens, T cell responses are not stimulated by self-antigens because B cells only express co-stimulatory molecules under conditions of bacterial stimulation or inflammation. This ensures that B cells only stimulate T cells under conditions of infection (Lanzavecchia, 1990).

Our *in vivo* experiments do not distinguish between types of pAPC, but focus simply on activating T cells *in vivo*. Our *in vitro* experiments used cultured bone marrow dendritic cells (BMDC). BMDC cultures contain B cells and macrophages although the majority of the cells present are DC.

1.3 Antigen Processing and Presentation

Understanding the pathways by which antigens are processed into peptides for presentation on MHC molecules is essential in order to efficiently activate T cells *in vivo*. Theoretically, access to diverse processing pathways will enhance the presentation of the antigen and ultimately enhance the activation of T cell immunity. Thus, what is currently known about the MHC molecules as well as the classical and non-classical antigen processing pathways will be reviewed.

1.3.1 MHC class I Antigen Processing and Presentation

1.3.1.1 MHC class I molecules

As shown in Figure 1.1**A**, MHC class I consists of two polypeptide chains, an α chain encoded within the MHC gene complex and a smaller, non covalently bound β_2-microglobulin chain, which is encoded on a separate chromosome. The α chain is made up of three domains, α_1, α_2 and α_3. Allelic variation is clustered in the α_1 and α_2 domains as these two domains fold together and create a long binding groove for peptides. α_1 and α_2 each form an α-helix and a β-pleated sheet. When the two domains associate with each other, the β-pleated sheet forms a platform on which the two α-helices rest. The α-helices form the sides of the peptide binding

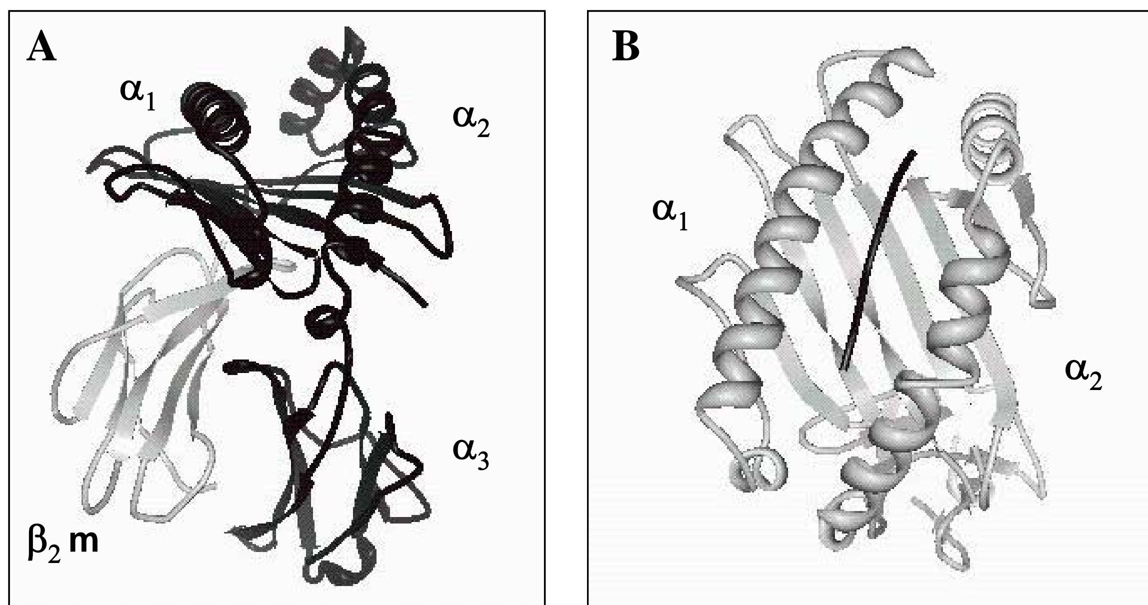

Figure 1.1 Structure of MHC Class I.

(A) MHC Class I molecule with the α chains shown in black and β_2-microglobulin shown in gray. (B) Peptide (black) in an MHC class I (gray) binding groove. Figures were generated using data submitted by Wilson et al (Fremont et al., 1992) to the RCSB Protein data bank, PDB number 2VAA. PDB file was then manipulated using the Protein Workshop on the RCSB Protein Data bank (Moreland et al., 2005).

groove while the β-pleated sheet forms the actual binding cleft (Figure 1.1B). Both α_3 and β_2-microglobulin are invariant and have a structure that closely resembles an immunoglobulin constant domain. β_2-microglobulin, unlike α_3 does not span the membrane, but is non-covalently associated with the base of the α_1 domain as well as with α_3. CD8 recognition of MHC class I occurs weakly at the invariant α_3 domain and at the base of the α_2 domain.

Upon synthesis of the MHC class I α chain and its insertion into the endoplasmic reticulum (ER), MHC class I binds to the ER membrane-associated chaperone calnexin and the thiol oxidoreductase ERp57 (Lindquist et al., 2001; Morrice and Powis, 1998; Zhang et al., 2006). Folding and disulfide bond formation between the domains occurs at this time, although calnexin is not necessary for proper folding to occur (Balow et al., 1995; Scott and Dawson, 1995). However, ERp57 accelerates the catalysis of the α_3 disulfide bond (Zhang et al., 2006). Association of β_2-microglobulin with the α chain releases calnexin and allows the chaperones involved in peptide loading to interact with MHC class I (Sadasivan et al., 1996). Peptide binding to MHC class I as well as how peptides access the ER will be discussed in detail as part of the classical MHC class I antigen processing.

1.3.1.2 Classical MHC class I Processing Pathway

The classical MHC class I processing pathway involves the processing of endogenous cytosolic proteins (Figure 1.2). As the vast majority of cells within the body express MHC class I, many self-peptides are presented. Responses against these self-peptides are rare as thymic selection deletes self-reactive CD8+ T cells. Self-reactive T cells that survive thymic selection are likely rendered anergic by recognition of self-antigen in the absence of co-stimulatory signals. Recognition of peptides presented on MHC class I allows CD8+ T cells to identify cells bearing

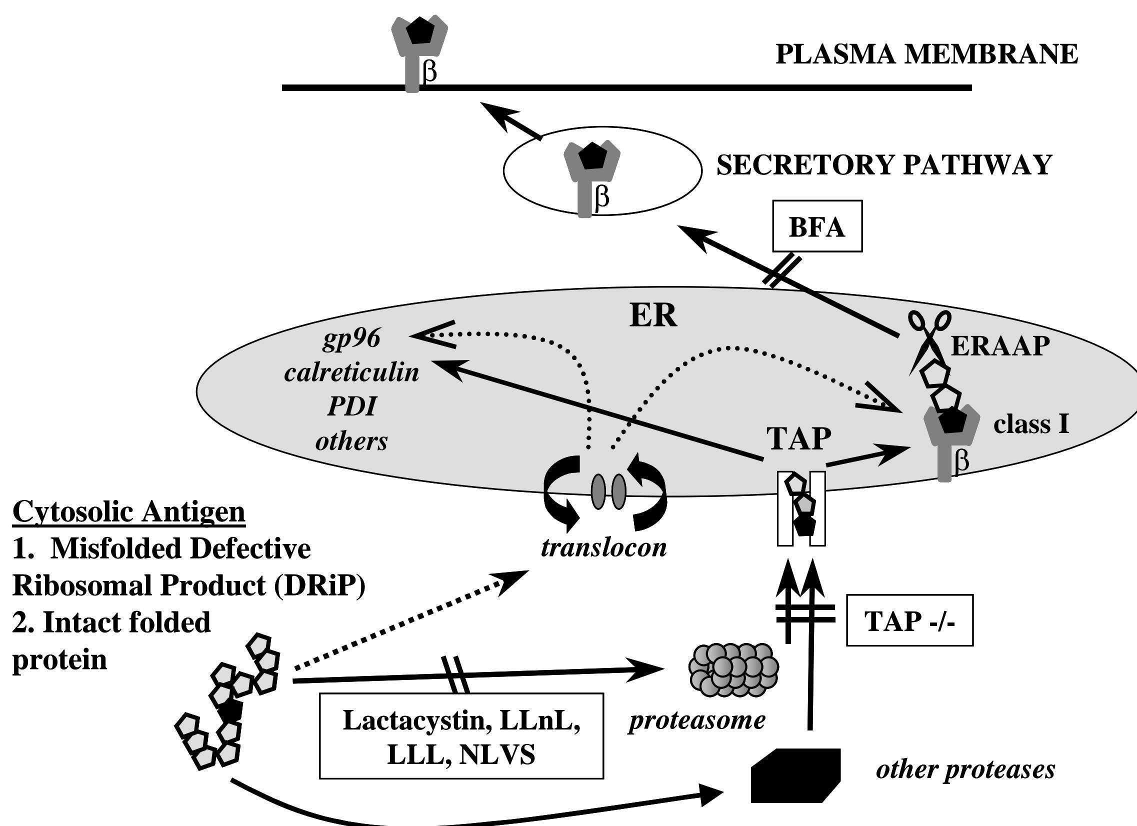

Figure 1.2 Overview of how an antigenic determinant (black pentagram) in a cytosolic antigen is loaded onto an MHC class I molecule.

Cytosolic antigens such as DRiPs and intact folded proteins are the source of antigens in the classical MHC class I processing pathway. The cytosolic antigen is degraded by the proteasome or other proteases (leucine amino-peptidase, furin, thimet oligopeptidase, and TPPII) in the cytosol before being transported into the ER via TAP. Alternatively, cytosolic antigens can be transported into the ER via the translocon. Antigenic peptides in the ER can either interact with MHC class I molecules or molecular chaperones such as gp96, calreticulin, PDI and others. The ER Aminopeptidase Associated with antigen Processing (ERAAP) trims N-terminally extended determinants either prior to after loading onto MHC class I. MHC class I is then transported to the cell surface via the secretory pathway. Short double lines through the pathway indicate manipulations that block the pathway. The specific manipulation that blocks the pathway is indicated by the boxed text. Adapted from (Yewdell et al., 1999).

foreign peptides, permitting CD8+ T cells to monitor cells for infection by viruses and intracellular bacteria. Tumor cells, which may express aberrant proteins can also be identified and eliminated in this manner.

1.3.1.2.1 Properties of MHC Class I Binding Peptides

Peptides that bind to MHC class I are generally 8-11 amino acids long and are anchored to the peptide binding groove at their amino and carboxy termini. The binding of the peptide is stabilized at each end of the peptide binding groove by invariant residues. A cluster of tyrosine or phenylalanine residues common to all MHC class I molecules forms hydrogen bonds with the free amino group of the peptide. The free carboxyl group on the carboxy terminus of the peptide is held in place by both hydrogen bonds and ionic interactions (Bouvier and Wiley, 1994; Madden et al., 1992). The length of the peptide is constrained to 8-11 amino acids because of the anchoring of the free amino and carboxyl groups at the terminus of the peptides. Longer peptides may bind, with the ends firmly anchored, but the middle of the peptide protrudes above the peptide binding cleft (Guo et al., 1992). Peptide protrusion from the middle of the binding cleft may prevent T cell receptor recognition of the MHC class I molecule and thereby prevent T cell receptor activation (Tynan et al., 2005a). However, some MHC class I allomorphs may allow for T cell recognition of a bulged peptide (Tynan et al., 2005b). Bulging peptides, however, are not a concern in our experiments which focus on the processing of the protein antigen ovalbumin. The binding of the 8 amino acid SIINFEKL peptide derived from ovalbumin to the mouse MHC class I molecule H-2K^b is well defined.

1.3.1.2.2 Origins of Proteins for Classical MHC I Processing

The substrate proteins in classical MHC class I processing are endogenous to the cell. Natural turnover of endogenous proteins was thought to be the main source of antigenic peptides. This idea was supported by the evidence that cells treated with degradation inhibitors, were

compromised in their ability to present antigenic peptides (Rock et al., 1994). In this model, recently synthesized proteins (such as viral proteins synthesized in the course of infection) or proteins with long half-lives would be poorly represented in the pool of peptides available for MHC class I binding. Yet experimental evidence demonstrated that CD8+ T cells could be activated by virally infected cells less than 60 minutes post infection (Esquivel et al., 1992), indicating that the natural degradation of endogenous proteins is not the only source of peptides for the MHC class I processing pathway.

Based on the observation that virally infected cells can be recognized within 60 minutes of infection, two possible sources were proposed for MHC class I peptides. One proposed source was short-lived, out-of-frame polypeptides resulting from errors in transcription or translation (Boon and Van Pel, 1989). A study by Boon *et al.* demonstrated that MHC class I can present cryptic translation products to T cells (Boon and Van Pel, 1989). This antigen source was confirmed by Shastri *et al.* when cryptic translation products derived from alternate initiation at leucine were also presented on MHC class I (Shastri et al., 1995). Others have since demonstrated that translation products from alternate open reading frames, introns, and intron-exon junctions are substrates for classical MHC class I processing in tumor recognition (Probst-Kepper et al., 2001; Rimoldi et al., 2000; Saeterdal et al., 2001; Wang et al., 1996). While errors in transcription and translation, as well as alternate translation, can serve as a source for MHC class I peptides, this source does not account for peptides derived from proteins translated in the correct reading frame.

The source of MHC class I peptides derived from proteins translated in the correct reading frame was proposed based on the observation that proteins modified for increased degradation resulted in increased presentation of the protein to CD8+ T cells (Tevethia et al., 1983; Townsend et al., 1988; Townsend et al., 1986a). This observation implies that imperfect forms of proteins generated from wild-type genes would be a preferred source of antigenic

peptides. Protein biosynthesis is error prone; in addition to errors that may arise from the generation of mRNA, protein synthesis itself can result in the mis-incorporation of amino acids, premature termination, or deletion of residues, resulting in imperfect proteins. Improper folding, post-translational modification or assembly of multi-protein complexes also result in imperfect proteins. Yewdell *et al.* collectively referred to all errors in protein biosynthesis as **D**efective **Ri**bosomal **P**roducts (**DRiP**s), and proposed that DRiPs were the major source of peptides for MHC class I processing and presentation (Yewdell et al., 1996). This hypothesis is supported by the observation that DRiPs are rapidly degraded (Reits et al., 2000; Schubert et al., 2000), explaining the original observation that virally infected cells could be recognized by T cells less than 60 minutes after infection (Esquivel et al., 1992). DRiPs were demonstrated to be defective proteins when immunoprecipitation of a long-lived protein from radio-labeled cells treated with proteasome inhibitors revealed multiple molecular weight bands for the long-lived protein. It was then demonstrated that the multiple molecular weights were due to polyubiquitination, signifying that these bands were likely defective proteins targeted for degradation (Schubert et al., 2000). The calculation that DRiPs constitute 30% of all synthesized proteins (Schubert et al., 2000) further strengthens the DRiPs hypothesis as it would permit presentation of peptides from newly synthesized bacterial and viral products, leading to the observed rapid presentation. Additionally, newly synthesized, ubiquitinated proteins were observed to transiently accumulate in aggregates during dendritic cell maturation, further strengthening the DRiPs hypothesis that newly synthesized rapidly degraded proteins are the source of peptides for MHC class I presentation (Lelouard et al., 2004; Lelouard et al., 2002). Together, these observations suggest that to optimally target an antigenic protein to the MHC class I processing pathway, the antigenic protein should be newly expressed and/or rapidly degraded. We examine the *in vivo* requirements for proteins to access the classical MHC class I processing pathway in Chapter 3.

1.3.1.2.3 Cytosolic Processing

Degradation of cytosolic protein is essential to the generation of antigenic peptides for MHC class I presentation. Degradation of proteins is tightly regulated to prevent non-specific destruction of essential self-proteins. Specific signals, such as PEST sequences (sequences rich in proline, glutamine, serine and threonine) (Rogers et al., 1986), destruction boxes (Glotzer et al., 1991), and ubiquitination (Townsend et al., 1988; Varshavsky, 1992) target proteins for degradation. The role of ubiquitination in the degradation of proteins for the classical MHC class I processing pathway was initially unclear as studies with ovalbumin generated conflicting results. Michalek *et al.* initially found that presentation of micro-injected ovalbumin was ubiquitin-dependent (Michalek et al., 1993). However, Cox *et al.*, demonstrated that ubiquitination of endogenously synthesized ovalbumin was not required for presentation on MHC class I (Cox et al., 1995). An additional study by Michalek *et al.* found that presentation of endogenously synthesized ovalbumin occurred in both an ubiquitin-dependent and an ubiquitin-independent manner but required proteasomal degradation (Michalek et al., 1996). This result suggests that degradation of antigen by the proteasome occurs in both ubiquitin-dependent and –independent manners. Recently, Qian *et al.* demonstrated that 75% of DRiPs were degraded in an ubiquitin-dependent manner by the 26S proteasome while the remaining 25% were degraded in a ubiquitin-independent manner by the 20S proteasome (Qian et al., 2006), further implicating both degradation pathways in MHC class I processing.

The discovery that expression of LMP2 and LMP7, proteasome subunits encoded within the MHC gene complex, is induced by IFN-γ, further implicated the importance of the proteasome in the degradation of cellular proteins to peptides for MHC class I presentation (Cerundolo et al., 1995; Ortiz-Navarrete et al., 1991). LMP2 and LMP7, together with another IFN-γ inducible proteasome subunit MECL-1 (Groettrup et al., 1997), make up the immunoproteasome, a proteasome with altered cleavage site preference (Driscoll et al., 1993;

Toes et al., 2001), allowing production of a wider range of peptides for presentation to the immune system. The immunoproteasome permits the generation of a wider range of MHC class I epitopes by improving the generation of some epitopes (Schwarz et al., 2000; Sijts et al., 2000a; Sijts et al., 2000b) and negatively influencing the generation of others (Morel et al., 2000; Van den Eynde and Morel, 2001).

Due to the difficulty of detecting proteolytic intermediates, it was thought that the proteasome or immunoproteasome generated antigenic peptides suitable for loading onto MHC class I (Dick et al., 1996; Niedermann et al., 1995). The development of a novel system by the Shastri group permitted the detection of antigenic precursors in both the cytosol and the ER (Paz et al., 1999). The system utilized several key modifications in the precursor protein sequence that allowed well-characterized proteases to make the appropriate N- (trypsin) and C- (carboxypeptidase B) terminal cuts to generate the antigenic peptide. Thus, after fractionating cell extracts by reverse-phase HPLC, naturally processed intermediates could be identified by enzymatically releasing the antigenic peptide and using it to stimulate a T cell hybridoma. Using this system, it was revealed that antigenic peptides with precise N- and C-terminal ends were not detected in the cytosol (Paz et al., 1999). However, C-terminally extended precursors were only detected under conditions of proteasome inhibition, implying that precise C-terminal ends are generated by the proteasome (Kunisawa and Shastri, 2003; Kunisawa and Shastri, 2006). N-terminally extended antigenic peptides were detected in the cytosol and associated with high molecular weight material that was likely chaperones (Paz et al., 1999). These N-terminally extended precursors were readily transported into the ER by the transporter associated with antigen processing (TAP), implying that the N-terminal residues were processed in the ER (discussed below) (Paz et al., 1999). The high molecular weight material associated with the N-terminally extended antigen was identified as the group II chaperone TRiC in a subsequent study (Kunisawa and Shastri, 2003). TRiC interacted with the N-terminally extended proteolytic

intermediates and prevented their degradation by cytosolic proteases (Kunisawa and Shastri, 2003). How TRiC acquires proteasome products and how the said products are then released for transport into the ER is unclear (Kunisawa and Shastri, 2003). A recent study demonstrated that large N- and C-terminally extended precursors were associated with another chaperone, hsp90α (Kunisawa and Shastri, 2006). Hsp90α associated only with the largest of the N- and C-terminally extended precursors, suggesting that hsp90α associated only with proteasomal precursors (Kunisawa and Shastri, 2006). Knockdown of hsp90α, or its co-chaperone CHIP, inhibited the recovery of antigenic peptide:MHC class I complexes on the cell surface, implying that hsp90α and CHIP direct the antigenic precursor to proteasomal degradation (Kunisawa and Shastri, 2006). Together with the TRiC data, this study suggests that chaperones are involved both in targeting antigenic proteins for initial proteolytic degradation as well as in protecting the proteolytic intermediates from degradation prior to transport into the ER.

The proteasome is not the only means of generating peptides for MHC class I in the cytosol. Proteasome inhibition does not abrogate all MHC class I presentation; inhibition of the proteasome can enhance the presentation of certain epitopes (Luckey et al., 1998; Vinitsky et al., 1997). Several other cytosolic proteases, such as leucine amino-peptidase (Beninga et al., 1998), furin (Gil-Torregrosa et al., 1998) and thimet oligopeptidase (Silva et al., 1999) have been implicated in the degradation of proteins for presentation on MHC class I. However, the selective cleavage specificities of these proteases can generate only a limited subset of peptides that are appropriate for MHC class I binding. It has been suggested that MHC class I epitopes carrying a lysine residue at the C-terminus are inefficiently produced by the proteasome (Benham et al., 1998). Thus, tripeptidyl protease II (TPPII), which exhibits endoproteolytic cleavage properties and is able to cleave after lysine residues (Geier et al., 1999), is a candidate protease for generation of epitopes the proteasome is unable to generate. TPPII has already been

demonstrated to generate an epitope from the HIV Nef protein that the proteasome is unable to produce (Seifert et al., 2003). A recent study by Kunisawa and Shastri, also demonstrated that TPPII and/or other serine proteases are able to generate the correct C-terminus of an MHC class I peptide (Kunisawa and Shastri, 2006). Nevertheless, TPPII is thought to be more involved in the trimming of large N-terminal extensions from long precursor peptides (Levy et al., 2002). MHC class I molecules bind peptides of 8-10 amino acids (Townsend et al., 1986b), yet proteasomal products have been demonstrated to be much larger, 16-22 amino acids, in length (Kisselev et al., 1999; Toes et al., 2001). Clearly, these proteasomal products undergo additional processing before they are an appropriate size for binding to MHC class I molecules. TPPII has aminopeptidase activity (Geier et al., 1999), and is able to trim both long and short N-terminal extensions (Levy et al., 2002; Reits et al., 2004; York et al., 2006). A recent study implies that TPPII processes longer proteasomal products (greater than 16 amino acids) until the product is short enough to become a substrate for other peptidases (York et al., 2006). Once the product becomes a substrate for other peptidases, the product is far more likely to be completely degraded than to be transported into the ER. Calculations by Yewdell (Yewdell, 2001) and experimental data from Montoya and Del Val (Montoya and Del Val, 1999) show that only one peptide binds to MHC class I for every ten thousand proteins degraded, suggesting that many peptides are degraded prior to transport into the ER. These calculations emphasize the necessity of targeting antigenic proteins to multiple processing pathways in order to enhance presentation of the antigen to CD8+ T cells. These calculations also have implications for our studies using different viral vectors to express the same antigen. In these studies, an antigen that can access both the proteasome as well as cytosolic proteases for degradation could theoretically have enhanced presentation over an antigen which can only access one of the degradation pathways.

1.3.1.2.4 Antigenic Peptide Transport into the ER

Transport of peptides generated in the cytosol into the ER is essential for the peptides to interact with MHC class I. Transport into the ER customarily (but not always) requires a special transporter, the transporter associated with antigen processing (TAP). TAP is a heterodimeric protein made up of TAP-1 and TAP-2. The TAP-1 and TAP-2 genes are encoded within the MHC gene complex (Monaco, 1992) and are confined to the ER (Kleijmeer et al., 1992). TAP transports 8-15 amino acid peptides from the cytosol into the ER in an ATP-dependent manner (Androlewicz et al., 1993; Koopmann et al., 1997; Neefjes et al., 1993; Shepherd et al., 1993). Both TAP-1 and TAP-2 contain a cytosolic ATP binding site (Kelly et al., 1992). To transport a peptide, the peptide must first bind to TAP. A peptide binding site is formed by the sequences of both TAP-1 and TAP-2 at a site between the pore and the ATP-binding site (Androlewicz and Cresswell, 1994; Nijenhuis and Hammerling, 1996; Nijenhuis et al., 1996). Peptide binding does not require hydrolysis of ATP (Androlewicz and Cresswell, 1994; Shepherd et al., 1993; van Endert et al., 1994), however, transport of the peptide from the cytosol into the ER is dependent on ATP hydrolysis (Androlewicz et al., 1993; Neefjes et al., 1993; Shepherd et al., 1993). Studies examining the peptide specificity of the TAP peptide-binding site have indicated that the peptide binding site of human TAP is more promiscuous than that of murine TAP. TAP from both species binds peptides of 8-15 amino acids (Momburg et al., 1994). However, mouse TAP has a strong preference for peptides with hydrophobic C-terminal amino acids while human TAP will also bind peptides with basic C-terminal amino acids (Androlewicz and Cresswell, 1994; Heemels and Ploegh, 1994; Neefjes et al., 1995; Uebel et al., 1995; van Endert et al., 1995; van Endert et al., 1994). Peptides longer than 8-15 amino acids may be transported through TAP, however the efficiency of translocation decreases relative to the increased size of the peptide (Koopmann et al., 1996).

TAP is not the only means of peptides gaining entrance to the ER. Cells lacking TAP are able to load peptides on MHC class I molecules, implying that alternate routes exist for peptides

to access the ER (Henderson et al., 1992; Wei and Cresswell, 1992). A number of possible mechanisms have been identified for TAP-independent presentation of antigenic peptides. The best characterized mechanism involves peptides liberated from signal sequences of membrane or secretory proteins in the ER(Henderson et al., 1992; Wei and Cresswell, 1992; Wolfel et al., 2000). Signal sequences target membrane proteins and secretory proteins into and across the ER membrane through the Sec61 translocon. After protein translocation, signal sequences are liberated in the ER by the signal peptidase enzyme (Meacock et al., 2000; Smith et al., 2002a). A second mechanism of TAP-independent presentation of endogenous protein involves epitopes located within the ectodomains of transmembrane proteins. These epitopes are not generated in a conventional way, but are liberated within the lumen of the ER by ER proteases (Elliott et al., 1995; Hammond et al., 1993; Snyder et al., 1997). A third potential mechanism of TAP-independent presentation of endogenous protein involves peptides generated by the trans-Golgi protease furin. This mechanism has been demonstrated primarily through epitopes engineered into transmembrane and secretory proteins, so the relevance of this mechanism to the presentation of natural proteins has yet to be determined (Gil-Torregrosa et al., 1998; Lu et al., 2004; Lu et al., 2001). In all of the potential mechanisms above, the pathway of presentation was not only independent of TAP, but independent of proteasomal processing as well.

The first example of a proteasome-dependent, TAP-independent pathway for endogenous antigen comes from the LMP2 protein of Epstein-Barr Virus (EBV). EBV LMP2 is unique from the LMP2 subunit of the immunoproteasome encoded in the MHC gene complex. EBV LMP2 is an integral membrane protein with short N- and C-terminal domains flanking 12 tandemly arranged transmembrane domains joined by tight loops showing minimal projection into the ER (Longnecker, 2000; Miller et al., 1995). TAP-independent processing of EBV LMP2 was initially reported for six out of the nine MHC class I epitopes of EBV LMP2 (Khanna et al., 1996; Lee et al., 1996). All of the EBV LMP2 epitopes were proteasome dependent, as inhibition of the

proteasome abolished EBV LMP2 epitope presentation (Lautscham et al., 2003a; Lautscham et al., 2001). Five of the six TAP-independent epitopes were then localized to the transmembrane regions of EBV LMP2 while the remaining epitope was localized to a cytoplasmic loop (Lautscham et al., 2003b). Surprisingly, after expressing the epitopes as minigenes using vaccinia virus, Latscham *et al.* discovered that the epitopes all retained their TAP-independency, suggesting that the TAP-independency was intrinsic to the epitope (Lautscham et al., 2001). Further investigation revealed that all of the TAP-independent epitopes were extremely hydrophobic (Lautscham et al., 2001). The mechanism by which these hydrophobic epitopes access the ER independent of TAP is unknown, however, it implies that hydrophobic epitopes have another mechanism to gain access to the ER.

Theoretically, enhancing the number of pathways by which an antigen can access the ER should enhance the presentation of that antigen. Thus, the above studies have important implications for our studies examining the ability of a virally expressed antigen to access different pathways of antigen processing *in vivo*.

1.3.1.2.5 Antigenic Peptide Processing in the ER

Two observations lead to the hypothesis that some peptide processing occurs in the ER. One, peptides liberated from a signal sequence could become ligands for MHC class I in TAP deficient cells (Hunt et al., 1992; Wei and Cresswell, 1992). Two, peptide ligands could be liberated from other ER-targeted proteins (Snyder et al., 1998; Snyder et al., 1994). The discovery that N-terminally extended peptides were present in the cytosol after proteasomal processing (Cascio et al., 2001) and that N-terminally extended peptides could be transported via TAP into the ER (Koopmann et al., 1996) further strengthened this hypothesis. The existence of an ER amino-peptidase was further supported by the ability of amino-peptidase inhibitors to block peptide trimming *in vitro* (Brouwenstijn et al., 2001; Fruci et al., 2001). It was later

determined that peptides with N-terminal extensions could be cleaved to the proper size for binding MHC class I in the ER, while peptides with C-terminal extensions could not (Serwold et al., 2001). The Shastri group then purified the ER amino-peptidase from microsomes isolated from mouse liver and spleen cells (Serwold et al., 2002). Saric *et al.* purified the human homolog at approximately the same time (Saric et al., 2002). This amino-peptidase was subsequently renamed ER Aminopeptidase associated with Antigen Processing (ERAAP). ERAAP was demonstrated to trim antigenic peptides to the required 8-10 amino acids in the ER in a size dependent manner (Chang et al., 2005). ERAAP binds to peptides of 9-16 amino acids in length, at the C-terminal residue hydrophobic side chains, similar to the binding of peptides to MHC class I (Chang et al., 2005). This localizes the active site of ERAAP nine amino acids away from the C terminus- the preferred length of an MHC class I peptide. Emphasizing its importance in processing peptides for presentation on MHC, ERAAP was demonstrated to be co-expressed with MHC in all mouse tissues and expression was up-regulated by IFN-γ (York et al., 2002). Inhibition of ERAAP activity led to decreased number of MHC-peptide complexes on the cell surface; emphasizing the importance of this amino-peptidase in antigen processing (Saric et al., 2002; Serwold et al., 2002). The Van Endert group recently discovered another human ER amino-peptidase, alternately called L-RAP or ERAP2. This amino-peptidase shares approximately 50% amino acid sequence homology with ERAAP and shares many of the same functions (Saveanu et al., 2005).

The discovery of ER processing of antigenic peptides raises the question about whether expressing minimal antigenic peptides is the best method for increasing presentation on MHC class I. Experiments in Chapter 3 will address this question for *in vivo* antigen presentation.

1.3.1.2.6 MHC I-peptide complex formation and transport to cell surface

After associating with β_2-microglobulin, MHC class I exchanges calnexin for calreticulin (Figure 1.3B) (Sadasivan et al., 1996). The calreticulin bound MHC class I molecule is then able to interact with TAP by binding to tapasin (Figure 1.3C) (Ortmann et al., 1994; Suh et al., 1994). This interaction between the MHC class I molecule and tapasin seems to function more to protect the MHC class I molecule from degradation than it does to enhance the capture of peptides by tethering MHC to TAP as soluble tapasin restores MHC class I function in a tapasin knock out cell lines (Lehner et al., 1998). However, it should be noted that differing requirements for tapasin have already been demonstrated for different MHC class I molecules (Grandea et al., 1997; Peh et al., 1998). ERp57 forms a bridge between calreticulin and tapasin as it is bound to both calreticulin and tapasin (Dick et al., 2002) (Figure 1.3B and C). Tapasin forms a similar bridge between MHC class I and the transporter associated with antigen processing-1 and -2 (TAP1 and TAP2) (Figure 1.3C). By tethering MHC class I to TAP1, tapasin places MHC class I in a optimal position to bind peptides as they enter the ER (Dick et al., 2002; Li et al., 1997; Ortmann et al., 1997). A study using a cell-free system revealed that ERp57and tapasin maximize the affinity of the peptide bound to MHC class I, indicating that ERp57 and tapasin constitute the functional component of the MHC class I peptide loading machinery (Wearsch and Cresswell, 2007). Binding of peptide completes the folding of MHC class I and releases it from calreticulin, tapasin and ERp57 allowing it to be transported to the cell surface (Figure 1.3D) (Ortmann et al., 1994; Suh et al., 1994). While peptide binding greatly increases the stability of MHC class I, MHC class I molecules receptive to binding exogenous peptide can be detected on the surface of TAP knockout cells, indicating that at least some MHC class I molecules are stable in the absence of peptide (Wei and Cresswell, 1992). Transport of MHC class I-peptide complexes proceeds through the Golgi and Trans Golgi Network to the cell surface, completing the classical MHC class I processing pathway and permitting presentation of antigen to CD8+ T cells (Neefjes and Ploegh, 1988). The time it takes for the MHC class I molecules to egress

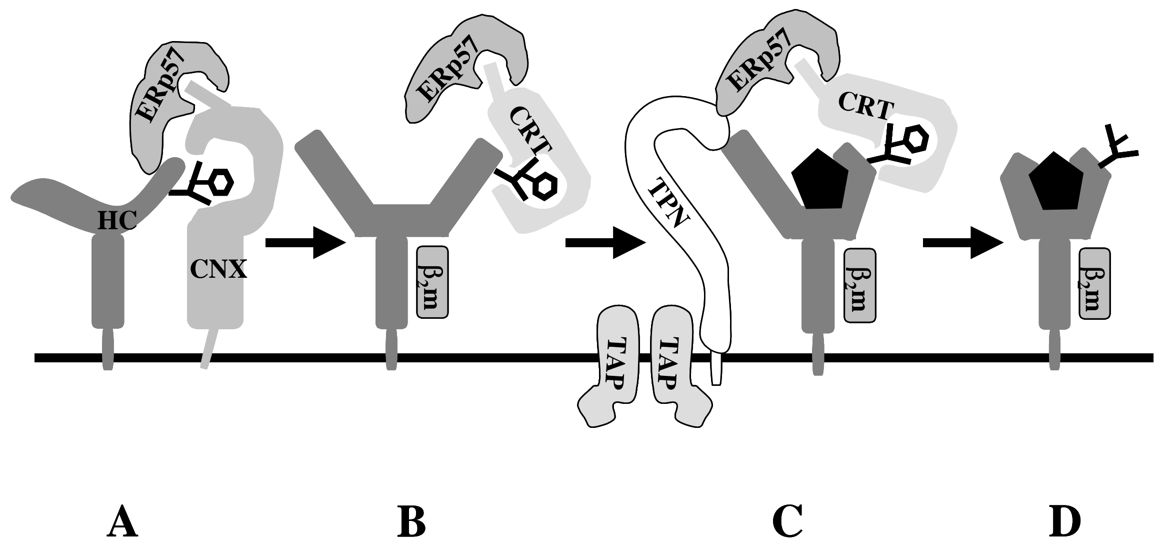

Figure 1.3 Overview of MHC class I loading and maturation.

Folding of the newly synthesized class I heavy chains (HC) (A) is supported by the transmembrane chaperone calnexin (CNX) and the thiol oxidoreductase ERp57. ERp57 accelerates the catalysis of the disulfide bonds. After binding of β2m, heavy chain-β2m heterodimers and ERp57 associate with calreticulin (B) and, subsequently, with the ensemble of accessory molecules called the loading complex (C). The loading complex contains ERp57, the transporter associated with antigen processing (TAP), the transmembrane protein tapasin (TPN), and the soluble chaperone calreticulin (CRT). The components of the loading complex work in concert to ensure binding of high affinity peptides to MHC class I heterodimers. Peptide-loaded class I molecules (D) leave the ER and are transported to the cell surface.

from the ER through the Golgi to the cell surface depends on the MHC class I molecule. H-2K ^{b} and D ^{d} molecules egress with a t $_{½}$ of approximately 20 minutes while H-2K ^{d}, D ^{b}, and L ^{d} require approximately 55 minutes to egress (Degen and Williams, 1991; Suh et al., 1994).

One caveat of the information presented in the previous paragraph is that this data is derived from the study of a limited number of MHC class I molecules. Several lines of evidence point to the fact that peptides are not loaded on MHC class I exclusively in the ER. Several MHC class I molecules, such as H-2D ^{b} (mouse) and HLA-B7 (human), have been demonstrated to associate with the invariant chain in the ER (Cerundolo et al., 1992; Vigna et al., 1996). The invariant chain, which possesses endosomal localization signals, directs MHC class I molecules to sites of MHC class II antigen processing, allowing for exogenous peptides to be presented on MHC class I molecules (Cerundolo et al., 1992; Sugita and Brenner, 1995; Vigna et al., 1996). Data from Tourne *et al.* demonstrating that splenocytes from knockout mice with targeted disruptions of both TAP and invariant chain assembled cell surface MHC molecules in amounts similar to TAP knockouts, arguing against a major role for invariant chain in MHC class I peptide loading (Tourne et al., 1996). This finding does not rule out MHC class I molecules acquiring peptides from the endosome, as MHC class I molecules were demonstrated to recycle in mouse macrophages, T cells, B cells and DC (Abdel Motal et al., 1993; Gromme et al., 1999; Hochman et al., 1991; Kleijmeer et al., 2001; MacAry et al., 2001; Reid and Watts, 1990). MHC class I molecules were also found to be internalized and recycled to the cell surface in human B cell lymphoblastoid cells, resulting in a low level of steady state MHC class I molecules in endosomes (Reid and Watts, 1990). The functional significance of these MHC class I molecules found in the endosome has yet to be determined. However, one study suggests that the function of MHC class I molecules in the endosome is to enable the presentation of endosomal antigens on MHC class I molecules (cross presentation) (Lizee et al., 2003). This study used a mouse MHC class I molecule, H-2K ^{b}, with a point mutation in the cytoplasmic tail to demonstrate that cross

presentation was abrogated when H-2K^b was unable to access the endolysosomal compartment in dendritic cells (Lizee et al., 2003). While this study is of particular interest to us because we use H-2K^b extensively in our experiments, it must be noted that the importance of the cytoplasmic tail of MHC class I molecules has only been demonstrated for this MHC class I molecule.

Further evidence that MHC class I molecules may be loaded with peptide outside of the ER comes from the observation that MHC class I molecules do not retain their peptide ligands uniformly. A substantial subset of MHC class I molecules lose their ligands within 30 minutes of their departure from the ER. These MHC class I molecules may have been loaded with a low-affinity peptide, or may not have optimally bound a peptide which would normally be of high affinity. Day *et al.* demonstrated that the dissociation of ligand may occur within the secretory pathway because empty MHC class I molecules receptive to binding peptides are detected in the Golgi by biochemical and immunocytochemical means (Day et al., 1995). MHC class I molecules lacking a high affinity peptide were shown to be denatured with a $t_{1/2}$ of approximately 5 minutes at 37ºC in the absence of β_2-microglobulin in the surrounding media (Day et al., 1995). The addition of β_2-microglobulin to the surrounding media stabilized the MHC class I molecules by at least an order of magnitude (Day et al., 1995) and were capable of binding exogenous peptides (Rock et al., 1990). This finding was thought to have implications for the presentation of exogenous proteins on MHC class I (Harding and Song, 1994; Song and Harding, 1996), as cells pulsed with exogenous peptide could stimulate T cell lines. However, the cells used as APC in these studies had a very high level of empty MHC class I due to paraformaldehyde fixation, which cross-links and stabilizes empty MHC class I (Rock et al., 1992). Subsequent studies demonstrated that pulsing cells with exogenous peptide was not a mechanism of MHC class I loading (Albert et al., 1998b; De Bruijn et al., 1995; Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1995; Rock et al., 1992; Schirmbeck and Reimann, 1996). This finding implies that exogenous peptides are unlikely to bind to peptide-receptive MHC class

I molecules available on the cell surface; exogenous peptides would need to be taken up by the cell in order to be presented on MHC class I, an observation that will be examined in our studies on non-classical MHC class I processing in Chapter 3.

1.3.1.3 Non-Classical MHC class I Antigen Processing Pathway (Cross-Priming)

The presentation of exogenous proteins on MHC class I was first observed *in vivo* by Michael Bevan in 1976 (Bevan, 1976). Bevan observed that mice immunized with fully allogenic cells, differing in both major and minor histocompatibility genes, generated CD8+ T cells specific for the minor histocompatibility antigens from the immunizing cells presented on recipient-MHC class I. This finding indicated that the minor histocompatibility antigens were somehow transferred from the immunizing cells in a way that permitted them to be presented by host cells. Additional studies by Bevan using radiation chimeras revealed that presentation of exogenous proteins on MHC class I molecules required bone marrow derived cells bearing self-MHC class I molecules (Bevan, 1977). These findings were expanded to include both Y chromosome-encoded antigens (Gordon et al., 1976) and a simian virus-40 encoded tumor antigen (Gooding and Edwards, 1980). This non-classical MHC class I presentation pathway was subsequently referred to as cross-priming when naïve T cells were activated or as cross-presentation when effector T cells, T cell lines or hybridomas were activated. New techniques and reagents were required to characterize the cellular basis for cross-priming as well as the processing and presentation pathways used by the APC to present exogenous antigen on MHC class I molecules.

1.3.1.3.1 Cellular Basis for Cross-Priming

The APC involved in cross-priming were first hinted at by the observation that cross-priming required bone marrow derived cells bearing self-MHC class I molecules (Bevan, 1977).

Macrophages were the first APC implicated in cross-presentation (Debrick et al., 1991; Kovacsovics-Bankowski et al., 1993; Rock et al., 1993). However, these cells may have been incorrectly identified as macrophages based on their phagocytic abilities (Debrick et al., 1991); at the time they were identified, it was not known that immature DC were phagocytic (Sallusto et al., 1995). B cells, which primarily take up antigen by receptor-mediated endocytosis, are unlikely to be the APC involved with cross-priming *in vivo* as it is unlikely that sufficient numbers of antigen-specific B cells are present during the primary immune response. However, B cells are capable of presenting exogenous antigen on MHC class I and may play a role in cross-priming antigen as part of a memory immune response (Hon et al., 2005; Ke and Kapp, 1996).

Isolation and identification of the APC involved in cross-priming proved to be difficult. Pulaski et al were the first to isolate APC and demonstrate that they facilitated cross-presentation *in vitro* (Pulaski et al., 1996). These APC were isolated by antibody depletion and demonstrated to be negative for antibodies staining for T cell and B cell markers; but were positive for molecules expressed on the cell surface of DC and macrophages. Chiodoni et al (Chiodoni et al., 1999) reported that CD11c+ dendritic cells infiltrating tumors transfected with the genes for GM-CSF and CD40 ligand were able to cross-present tumor antigens. However, these results should be interpreted with caution as tumor debris and infiltrating dendritic cells were incubated for long periods of time as a result of the purification process, potentially allowing antigen loading of the APC under these conditions. A separate report suggested that CD11c+ APC from the pancreas of mice transgenically expressing TNF-α in their islet β cells were able to present islet antigens to CD8+ T cells (Green et al., 2000). Nevertheless, the authors did not prove that these cells were responsible for cross-presentation of the islet antigens. A commonality of each of these reports is the isolation of APC from inflammatory tissue, raising the possibility that inflammation may be necessary for cross-priming to occur *in vivo*.

More recent attempts to identify the APC involved with cross-priming have been more successful. Macrophages were eliminated as candidate APC after it was demonstrated that while macrophages are able to take up exogenous antigen and cross-present it to CD8+ T cells, they do so inefficiently, with less than 5% of the cells presenting the exogenous antigen (Mitchell et al., 1998; Norbury et al., 1995; Rock et al., 1990). DC however, efficiently took up exogenous antigen in their immature state *in vitro* and were able to process it and present it to CD8+ T cells *in vitro* upon maturity (Albert et al., 1998b; Mitchell et al., 1998). *In vivo* studies then demonstrated that CD8+ DC, but not CD8- DC were responsible for cross-priming both cell-associated exogenous antigen (den Haan et al., 2000; Iyoda et al., 2002) and soluble exogenous antigen (Pooley et al., 2001). Furthermore, it has been demonstrated that some subsets of dendritic cells are constitutively able to cross-present antigens (den Haan and Bevan, 2002; den Haan et al., 2000; Pooley et al., 2001; Schulz and Reis e Sousa, 2002) while other subsets require activation by immune complexes (den Haan and Bevan, 2002), T cell help (Machy et al., 2002), toll-like receptor ligands (Datta et al., 2003) or other undefined signals (Delamarre et al., 2003). The reasons behind these differential requirements are unknown and remain to be elucidated for this pathway.

The implication that inflammation may be necessary for cross-priming to occur is potentially very important to our studies. Our studies examining the requirements of the cross-priming pathway *in vivo* are carried out under conditions of viral infection, providing inflammatory conditions. Our other studies utilize different viruses to express the same antigen. By expressing the antigen using a viral vector, we should essentially create the inflammatory environment necessary for cross-priming to occur.

1.3.1.3.2 Processing Pathways Available to Exogenous Antigens

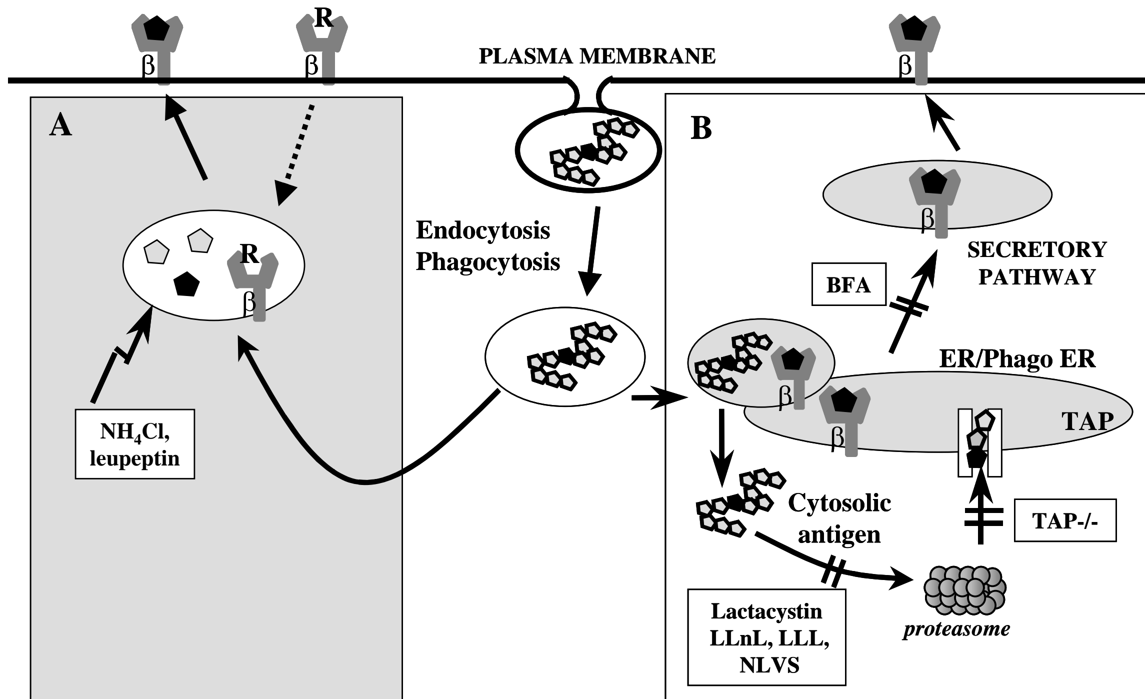

Figure 1.4 Processing pathways available to exogenous antigens for presentation on MHC class I.

In the endosomal (A), or TAP-independent, pathway of exogenous antigen processing, the antigen is internalized and degraded within the endocytic compartments to produce antigenic peptides able to bind post-Golgi or recycled MHC class I molecules. The dashed line shows recycled MHC class I molecules that are receptive, as indicated by the R in the peptide binding groove, to binding peptide. The boxed text indicates ways in which endosomal processing can be disrupted. In the cytosolic (B), or TAP-dependent, pathway of exogenous antigen processing, the antigen is released from the endocytic vesicles to the cytosol. Processing of the antigen then occurs by mechanisms of the classical MHC class I processing pathway. The short double lines through the pathway indicate manipulations that block the pathway. The specific manipulation that blocks the pathway is indicated by the boxed text. Adapted from figure created by Christopher Norbury.

Two processing pathways are available to exogenous antigens for presentation on MHC class I: endosomal and cytosolic (Figure 1.4). Endosomal processing of antigens for MHC class I presentation occurs in a similar manner to that of the classical MHC class II pathway, antigen is internalized and degraded within the endocytic compartments to produce antigenic peptides able to bind post-Golgi or recycled MHC class I molecules (Figure 1.4A). Endosomal processing requires endocytosis for uptake of antigen, low endosomal pH for activity of resident proteases, and endosomal proteases for processing of antigens (Harding and Song, 1994; Schirmbeck et al., 1995; Song and Harding, 1996; Yewdell et al., 1999). Inhibition of any of the requirements abrogates presentation on MHC class I. Cytosolic processing (Figure 1.4B) essentially utilizes the classical MHC class I presentation machinery. This means that cytosolic processing requires proteasome function for degradation of antigens; TAP function for the transport of peptides into the ER (unless, of course, the peptide enters the ER through a TAP-independent mechanism); and secretory function to ensure delivery of MHC class I-peptide complexes to the cell surface (Brossart and Bevan, 1997; Kovacsovics-Bankowski and Rock, 1995; Shen et al., 1997; Yewdell et al., 1999). Similar to the endosomal processing, inhibition of any of these cytosolic processing functions decreases presentation of antigens dependent on cytosolic processing. Assuming the requirement for TAP in the cytosolic processing pathway, the processing pathways can be distinguished from one another *in vivo* due to the availability of TAP knockout mice. Thus, our *in vivo* experiments use TAP knock mice in order to probe the antigen processing pathways.

1.3.1.3.2.1 Endosomal Processing

Since non-classical presentation on MHC class I utilizes exogenous antigen, it was thought that this pathway would use processing mechanisms similar to those of classical MHC class II processing. Indeed, cross-presentation of certain antigens was TAP-independent and

proteasome-independent (Liu et al., 1995; Martinez-Kinader et al., 1995; Schirmbeck et al., 1995; Schirmbeck and Reimann, 1994; Song and Harding, 1996; Wick and Pfeifer, 1996). The precise location at which the antigenic peptides are processed in this endosomal pathway is unclear, but there is evidence that antigens may be generated in the phagolysosomal or endosomal compartments as lysosomotrophic agents block presentation (Liu et al., 1997; Martinez-Kinader et al., 1995; Schirmbeck et al., 1995; Schirmbeck and Reimann, 1994). Endosomal processing of soluble ovalbumin was blocked by leupeptin treatment, suggesting that serine proteases such as cathepsins were involved in the endosomal processing of antigens for presentation on MHC class I (Shen et al., 2004). Cathepsin S, and not cathepsins B, D and L, was later demonstrated to be necessary for the endosomal processing of and subsequent presentation of ovalbumin as well as two influenza viruses epitopes (Shen et al., 2004). The involvement of cathepsin S was further confirmed *in vivo* when mice lacking both TAP and cathepsin S were unable to mount a CD8+ T cell response to either ovalbumin or influenza epitopes (Shen et al., 2004). Cathepsin S was demonstrated to be sufficient to process ovalbumin to the antigenic peptide SIINFEKL *in vitro*, although precisely how cathepsin S cleaves ovalbumin to produce the SIINFEKL peptide is unknown; the SIINFEKL peptide may simply be fortuitously flanked by cathepsin S cleavage sites (Shen et al., 2004). Yet, it seems highly unlikely that a single protease could precisely generate both the N- and C-termini favored for MHC class I binding; classical MHC class I processing requires proteasomal degradation, cytosolic proteases processing as well as ER processing in order to generate precise MHC class I binding epitopes. A more likely scenario is that endosomal processing generates a different pool of MHC class I binding peptides (Schirmbeck et al., 1998). However peptides are generated in the endosomal pathway, different mechanisms have been proposed for the loading of these peptide onto MHC class I molecules in the endosome, including peptide regurgitation, peptide exchange, and ER fusion with phagosomes.

Regurgitation of endosomal peptides into the extracellular fluid where they could interact with peptide-receptive MHC class I molecules was proposed by Song and Harding (Harding and Song, 1994; Song and Harding, 1996). However, the APC used in their studies had an abnormally high level of peptide-receptive MHC class I molecules on the cell surface due to paraformaldehyde fixation, which cross-links and stabilizes peptide-receptive MHC class I (Rock et al., 1992) and this mechanism was subsequently refuted (Albert et al., 1998b; De Bruijn et al., 1995; Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1995; Rock et al., 1992; Schirmbeck and Reimann, 1996). Observations that newly synthesized MHC class I molecules reach the endosomal compartments through direct transport form the ER (De Bruijn et al., 1995) or from internalization from the cell surface (Abdel Motal et al., 1995; Abdel Motal et al., 1993; Capps et al., 1989; Dasgupta et al., 1988; Gromme et al., 1999; Hochman et al., 1991; Machy et al., 1987; Reid and Watts, 1992; Tse and Pernis, 1984; Vega and Strominger, 1989) resulted in the hypothesis that peptides bound to these MHC class I molecules could be exchanged for endosomally generated peptides due to the low pH present in endosomes (Gromme et al., 1999; Hochman et al., 1991; Stryhn et al., 1996). However, this mechanism has never been demonstrated to be involved in cross-presentation, suggesting that another mechanism is used to load endosomally generated peptides onto MHC class I molecules.

In DC, the ER can fuse with the phagosome, permitting MHC class I molecules to access peptides generated in the phagosome (Gagnon et al., 2002). The presence of ER aminopeptidase, ERAAP, has also been demonstrated in phagosomes, permitting the trimming of peptides for loading onto MHC class I (Ackerman et al., 2003). Together these observations suggest that in pAPC, the classical MHC class I machinery can be found in phagosomes, permitting the endosomally generated peptides to be presented on MHC class I molecules and further substantiating a role for specialized cells in cross-priming.

1.3.1.3.2.2 Cytosolic Processing

The observation that chloroquine failed to inhibit MHC class I presentation of some exogenous antigens indicated that the antigenic peptides were not exclusively produced in endolysosomal compartments (Jin et al., 1988). The additional requirements for new synthesis of MHC class I, TAP and proteasome activity further suggested that some exogenous antigens could gain access to the cytosol and the classical MHC class I processing pathway (Brossart and Bevan, 1997; Kovacsovics-Bankowski and Rock, 1995; Liu et al., 1995; Norbury et al., 1997; Norbury et al., 1995; Reis e Sousa and Germain, 1995; Shen et al., 1997). Several mechanisms have been proposed to explain how exogenous antigens gain access to the cytosol from the phagosome. The loss of phagosome membrane integrity due to an excess of antigen (Reis e Sousa and Germain, 1995) and the manipulation of the phagosome by bacteria resulting in the release of phagosome contents into the cytosol (Brunt et al., 1990; Darji et al., 1995; Mazzaccaro et al., 1996) are two well-defined mechanisms by which exogenous antigen can access the cytosol. The observation of ER membranes being donated to nascent phagosomes during phagocytosis led to the hypothesis that antigens were retrotranslocated from the phagosome into the cytosol (Ackerman et al., 2006; Desjardins et al., 1994; Gagnon et al., 2002; Guermonprez et al., 2003). Access to the cytosol is provided through retrotranslocation of antigens presumably through the Sec61 pore complex. Retrotranslocation of misfolded proteins from the ER into the cytosol through the Sec61 pore complex is well established (Pilon et al., 1997; Wiertz et al., 1996). Although the main function of Sec61 is to insert secretory or transmembrane proteins into the ER, Sec61 has been demonstrated to also translocate proteins into the ER (Pilon et al., 1997; Wiertz et al., 1996). Sec61-mediated retrotranslocation removes unstable or accumulating proteins from the ER for proteolysis by the proteasome(McCracken and Brodsky, 2003). Thus, ER-membrane fusion with phagosomes would provide exogenous antigens with access to Sec61, permitting exogenous antigens to be degraded in the cytosol. Soluble exogenous antigens may also use

retrotranslocation to access the cytosol. DC can internalize soluble exogenous antigens and transport them into the ER (Ackerman et al., 2005; Imai et al., 2005). While this route may be limited to DC, it further strengthens the idea that specialized cells are required for cross-priming *in vivo*.

1.3.1.3.3 Antigen Uptake in Cross-Priming Pathway

Antigen uptake in the cross-priming pathway occurs by macropinocytosis, phagocytosis and receptor mediated endocytosis, similar to classical MHC class II antigen uptake. However, several studies have demonstrated that the route of endocytosis can affect the processing pathways available to the antigen.

Macropinocytosis is a constitutive process by which APC sample their extracellular environments. Macropinocytosis was demonstrated to deliver exogenous proteins to the cytosol of both macrophages and DC (Norbury et al., 1997; Norbury et al., 1995). Blocking macropinocytosis in these cells prevented both cytosolic delivery of exogenous antigens and antigen presentation (Norbury et al., 1997; Norbury et al., 1995). Antigen presentation of the exogenous antigens was dependent on cytosolic processing as antigen presentation was TAP-dependent and sensitive to proteasome inhibition as well as to inhibition of the secretory pathway (Norbury et al., 1997; Norbury et al., 1995). Chloroquine treatment, which inhibits function of the endocytic pathway, enhanced antigen presentation, suggesting that endosomal processing is not necessary for the generation of peptides from antigens taken up by macropinocytosis (Accapezzato et al., 2005). This implies that exogenous antigens taken up via macropinocytosis are processed primarily by cytosolic processing. The enhanced presentation after chloroquine treatment further suggests that chloroquine treatment increases the antigen available for transport into the cytosol for processing.

Targeting antigens to specific routes of uptake allowed examination of the processing pathways available to targeted antigens. Rock *et al.* compared the processing of soluble ovalbumin to that of particulate ovalbumin. Soluble ovalbumin was not presented on MHC class I in contrast to ovalbumin adsorbed to microspheres (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996). Additional studies with soluble proteins concurred with the findings of Rock *et al.*: that soluble protein was only cross-primed when very high concentrations of soluble protein were present (Kovacsovics-Bankowski et al., 1993; Norbury et al., 1995; Rock et al., 1990). Together, these studies implied that phagocytosis of particulate antigen was more efficient at producing MHC class I-peptide complexes than pinocytosis of soluble antigen. In a study examining the importance of the pathways of phagocytosis and pinocytosis in the cross-presentation of soluble antigen, Reis e Sousa *et al.* demonstrated that at a constant concentration of external antigen, antigen internalized by phagocytosis was cross-presented more efficiently than antigen internalized by pinocytosis (Reis e Sousa and Germain, 1995). This study implies that it is the route of endocytosis, rather than the form of antigen, that catalyzes efficient cross-presentation.

Other studies examined the efficiency of receptor-mediated endocytosis pathways in the processing of exogenous antigen. One such study looked at the ability of membrane-bound IgG on B cells to mediate internalization and cross-priming in the cross-presentation pathway (Ke and Kapp, 1996). In this study, the cross-presentation of soluble ovalbumin was compared to that of ovalbumin recognized by membrane-bound IgG (Ke and Kapp, 1996). Receptor-mediated endocytosis allowed internalization of the complex of immunoglobulin and ovalbumin. The authors found that cross-presentation of the immunoglobulin-ovalbumin complex was far more efficient than that of the soluble ovalbumin, with three to four magnitudes less ovalbumin needed to stimulate cross-presentation when it was bound by IgG (Ke and Kapp, 1996). This study also demonstrated that the IgG-bound ovalbumin reached the cytosol and was processed according to

the classical MHC class I pathway; it was both proteasome dependent and TAP dependent (Ke and Kapp, 1996). A study by an alternate group using Fc-receptor mediated endocytosis confirmed these results (Regnault et al., 1999), suggesting that targeting an antigen to the receptor-mediated endocytosis pathway permits the antigen to be processed in the cytosol for presentation on MHC class I.

Two studies targeted antigen to scavenger receptors for cross-presentation. The first study linked a heat shock protein (hsp) from *E. coli* to an extended ovalbumin peptide (18 amino acids) (Tobian et al., 2004) and examined the processing pathways available to ovalbumin in macrophages and dendritic cells. This study demonstrated that targeting ovalbumin to CD91, the α2-macroglobulin receptor, resulted in cytosolic processing of ovalbumin in dendritic cells but endosomal processing in macrophages (Tobian et al., 2004). In the second study, a soluble heat shock protein from *Mycobacterium bovis* was also linked to ovalbumin. The ovalbumin used in this study, however, was 110 amino acids in length and contained both the H-2^b MHC class I and I-A^b MHC class II ovalbumin determinants. This study demonstrated that the majority of internalized protein was transported to the lysosomes while the small fraction destined to be cross-primed exited from the endocytic pathway (Palliser et al., 2005). This small fraction required proteasomal activity and TAP for presentation on MHC class I (Palliser et al., 2005). The targeting of the proteins to the receptor-mediated endocytosis pathways allowed for increased cross-presentation compared to proteins internalized by phagocytosis. This further implies that targeting an antigen to a receptor may enhance the cross-presentation of the antigen.

Another study also targeted ovalbumin to the scavenger receptors by giving ovalbumin a negative charge due to modification with succinylation (Suc-OVA), maleylation (Mal-OVA) or *cis*-aconitylation (Aco-OVA) (Shakushiro et al., 2004). Similar to the results in the previous study, Takajura *et al.* discovered that maleylation or cis-aconitylation enhanced the cross-presentation of ovalbumin while succinylation did not. This study demonstrated that although the

Suc-OVA was targeted to the lysosome/endosome in the same manner as the Mal-OVA or Aco-OVA, it was not hydrolysed, preventing it from being degraded even if it did reach the cytosol; emphasizing that when targeting an antigen to a receptor through chemical modification, the pathway of modification needs to be considered. (Shakushiro et al., 2004)

Together these studies suggest that the route of endocytosis affects the processing pathways available to the antigen. This is of particular interest to our studies examining the antigen processing pathways available to an antigen when expressed by different viral vectors. Any differences observed in the ability of the antigen to be cross-primed may not be due to viral expression of the antigen alone, but due instead to the routes of endocytosis available to the antigen.

1.3.1.3.4 Antigenic Substrates for Cross-Priming

A major focus of our studies will be the form of antigen transferred to APC *in vivo*. Knowledge of the form of antigen transferred *in vivo* is necessary to enhance activation of CD8+ T cells by vaccination. *In vitro* studies have utilized many different types of antigens, including particulate antigens, viral antigens, soluble antigens and degradation intermediates as substrates to probe the processing and presentation requirements of non-classical MHC class I presentation. For each of these types of antigens, it was determined that the context of the antigen as well as the cell type used for processing dictated the processing and presentation pathways available to that antigen. Because these studies use differing antigen types as well as differing APC, comparing the results of the studies is incredibly complicated. Thus, in an effort to simplify the comparison, these studies will be discussed by the type of antigenic substrate.

1.3.1.3.4.1 Particulate Antigens

Bacteria have been utilized as particulate antigens to study the presentation of exogenous proteins on MHC class I. Expression of the immunodominant epitope of ovalbumin, $OVA_{257\text{-}264}$

in the context of a cytoplasmic, periplasmic or exoplasmic fusion proteins in E. coli demonstrated that $OVA_{257-264}$ was presented by macrophages exposed to the manipulated bacteria regardless of the protein context (Pfeifer et al., 1993). Presentation required phagocytosis as presentation was blocked by cytochalasinD and was not detected in either a non-phagocytic cell line or in macrophages exposed to the soluble proteins rather than the particulate bacteria. Additional experiments with the cytoplasmic protein, Crl-OVA, demonstrated that the macrophage presentation of $OVA_{257-264}$ was enhanced by addition of exogenous β_2-microglobulin and was insensitive to proteasome inhibitors; implying that $OVA_{257-264}$ was processed outside the cytosol and within the endosomes (Song and Harding, 1996). Presentation of $OVA_{257-264}$ from Crl-OVA required 30 fold more antigen in TAP-deficient macrophage, although this was demonstrated to be an effect of the decreased availability of MHC class I molecules on the cell surface rather than a requirement for ER delivery of the peptides (Song and Harding, 1996). In contrast to the presentation of Crl-OVA by macrophages, a phagocytic epithelial cell line was unable to generate detectable amounts of $OVA_{257-264}$ from Crl-OVA, despite the fact that it could readily present synthetic $OVA_{257-264}$ (Harding and Pfeifer, 1994). This further implies that specialized cells are required for cross-presentation of antigens. This specialized function of macrophages was then confirmed when macrophages, but not DC were able to process Crl-OVA by endosomal processing (Svensson and Wick, 1999). These findings indicate that proteins from phagocytosed bacteria are preferentially processed in the endosomes of macrophages. One caveat to these studies is that the use of an engineered determinant ($OVA_{257-264}$) that is normally produced from cytosolic processing (Wick and Pfeifer, 1996) may bias this system towards cytosolic processing. Thus, the lack of endosomal processing of Crl-OVA in DC may actually reflect this bias, rather than a lack of function for DC endosomes. Future studies utilizing bacterial, rather than engineered, determinants would aid in defining the endosomal pathway in both macrophages and DC.

Artificial particles have also been generated to study presentation of exogenous antigens on MHC class I. While soluble ovalbumin was not cross-primed *in vivo* (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996), work with bacteria suggested that particulate antigen may be favored for presentation by MHC class I. Thus, ovalbumin was coupled to either iron or latex beads in order to determine if this made ovalbumin accessible to presentation by MHC class I. Coupling ovalbumin to beads increased the efficiency of $OVA_{257-264}$ presentation by 3-4 orders of magnitude, and inhibitor studies further demonstrated that the presentation of $OVA_{257-264}$ was dependent on phagocytosis (Kovacsovics-Bankowski et al., 1993). Additional studies using ovalbumin coupled to beads demonstrated that the presentation of $OVA_{257-264}$ from both macrophage and dendritic cell lines was dependent on cytosolic processing as presentation required TAP and was sensitive to proteasome inhibitors (Kovacsovics-Bankowski and Rock, 1995; Shen et al., 1997). However, when Harding and Song conjugated ovalbumin to latex beads, they found that processing occurred in a cytosol-independent manner (Harding and Song, 1994; Song and Harding, 1996). Reis e Sousa and Germain demonstrated that soluble ovalbumin added to cells in the presence of latex beads was presented by macrophages and any other cell capable of phagocytosing latex beads in a cytosolic processing-dependent manner (Reis e Sousa and Germain, 1995). These findings imply that phagocytosis of the beads resulted in the transfer of the endosomal contents to the cytosol, not just proteins coupled to the beads. Thus, three different groups using artificial particles revealed that both cytosolic and endosomal processing of antigens coupled to beads were possible (Harding and Song, 1994; Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1995; Song and Harding, 1996; Svensson and Wick, 1999; Wick and Pfeifer, 1996). The discrepancies in these results are most likely due to differences in the methodologies used in each study. Differences in how the macrophages used in the studies were generated, the size of the beads and

the method of coupling used to attach the antigen to the bead could all have produced the contrasting results.

Cellular debris from apoptotic cells is another form of particulate antigen used to study the processing and presentation of exogenous proteins on MHC class I. Because macrophages have the ability to recognize and phagocytose apoptotic cells (Savill et al., 1993) and since cell death from apoptosis and necrosis is common at sites of inflammation, dead cells are thought to be a major source of exogenous antigen. Bellone *et al.* demonstrated that macrophages were able to phagocytose virally transformed apoptotic cells and present viral antigens (Bellone et al., 1997). Material from biotinylated apoptotic cells could be detected via microscopy using fluorescent streptavidin. Radiolabeled material from apoptotic cells incubated with radioactive isotopes could also be recovered from cytosolic fractions, further demonstrating the release of material from apoptotic cells into the cytosol. Presentation of antigen derived from apoptotic cells was TAP-dependent and required the synthesis of MHC class I (Bellone et al., 1997), demonstrating cytosolic processing of antigens derived from apoptotic cells. Another study utilizing apoptotic cells as the antigenic substrate found that DC could phagocytose virally-infected apoptotic cells and present epitopes from viral antigens (Albert et al., 1998b). This group further determined that the antigenic substrate of apoptotic cells was located in the cellular debris. These two studies indicate that phagocytosis of apoptotic cells results in the generation of MHC class I peptide complexes.

The cross-presentation of cellular debris from necrotic cells was compared to that from apoptotic cells in a study by Fonteneau *et al.* (Fonteneau et al., 2003). No difference was seen in the cross-presentation of antigens derived from cellular debris, regardless of the source (Fonteneau et al., 2003). Antigens derived from cellular debris were actively internalized and preprocessed in endosomes by cathepsinD. Antigen was processed in a proteasome- and TAP-

dependent manner (Fonteneau et al., 2003). This study demonstrates that both antigens from apoptotic and necrotic cells are cross-presented equally by DC *in vitro*.

The studies examining the cross-presentation of cellular debris are of particular interest to our *in vivo* studies using multiple viruses to express the same antigen. One of the viruses used in this study is cytopathic, which may result in an increase in substrate for the cross-priming pathway.

1.3.1.3.4.2 Viral Antigens

Our studies focus on the processing and presentation pathways available to virally expressed antigens, thus the *in vitro* studies using viral antigens are particularly relevant to our work. The presentation of viral proteins from inactivated viral particles was demonstrated for Sendai virus (Koszinowski et al., 1977; Schrader and Edelman, 1977). Endosomal processing was then demonstrated for the immunodominant epitope of Sendai virus by the presentation of this epitope in the absence of both TAP and the secretory pathway (Zhou et al., 1993a; Zhou et al., 1993b). Inactivation of the fusion activity of Sendai virus glycoproteins by heat did not abrogate the ability of the immunodominant epitope to be presented in cell lacking either the cytosolic processing pathway or the endosomal pathway, implying that heat inactivated Sendai virus can access both pathways (Liu et al., 1997; Liu et al., 1995). Cross-presentation of inactivated viruses was also demonstrated for influenza A virus (IAV), human cytomegalovirus (HCMV), and human immunodeficiency virus (HIV) *in vitro* (Vinitsky et al., 1997; Yewdell and Bennink, 1989; Yewdell et al., 1988). The presentation of IAV, HCMV, and HIV virion proteins was not dependent on viral gene expression, but involved the processing of virion proteins introduced to the cell as a result of viral fusion with the cell membrane (Buseyne et al., 2001; Riddell et al., 1991; Yewdell et al., 1988). Antibody staining revealed that IAV proteins accumulated in the cytosol of fibroblasts (Yewdell et al., 1988). Presentation of proteins from

both inactivated IAV and HCMV was abrogated with Brefeldin A treatment, suggesting that presentation of virion proteins required nascent MHC class I (Riddell et al., 1991; Yewdell and Bennink, 1989). While the processing of HIV and HCMV virion proteins was not probed, cytosolic processing was demonstrated for IAV polymerase 1 and nonstructural protein 1 (Vinitsky et al., 1997). However, IAV hemagglutinin and nucleoprotein, were presented in a proteasome-independent manner (Vinitsky et al., 1997) despite the fact that they are readily detected in the cytosol (Yewdell et al., 1988). This suggests that cytosolic proteases other than the proteasome process IAV hemagglutinin and nucleoprotein. Together these studies suggest that the cross-presentation of virion proteins may be an important mechanism by which CD8+ T cells are activated, although this remains to be probed *in vivo*.

In addition to viruses, virus-like particles have been used as substrates to examine the requirements for exogenous antigen presentation by MHC class I. Virus-like particles are composed of self-assembling proteins and have been demonstrated to induce CD8+ T cell responses *in vivo*. Study of the non-classical MHC class I processing and presentation of virus-like particles has been limited to Hepatitis B Virus (HBV) S particles. S particles form in the ER of HBV infected cells as a normal part of the infection and are composed of an S protein complexed with glycolipid. The ability of S particles to elicit protective immunity is emphasized by the fact that S particles were the basis of the HBV vaccine. Presentation of S particles *in vitro* occurred in the absence of both TAP and the secretory pathway, suggesting that S particle processing does not occur by cytosolic processing, but by endosomal processing (Schirmbeck et al., 1995). It should be noted that no controls demonstrated the efficacy of the Brefeldin A used to inhibit the secretory pathway. However, endosomal processing of S particles is supported by the observation that S particle presentation was blocked upon addition of leupeptin, an inhibitor of endosomal proteases. The absolute requirement for β_2-microglobulin in the presentation of S particles (Schirmbeck et al., 1997) demonstrates that S particle derived peptides bind to peptide-

receptive or “empty” MHC class I molecules either on the cell surface or in the endosomal pathway. These findings suggest that presentation of S particles involves the loading of endosomally generated peptides on MHC class I molecules bearing exogenous β_2-microglobulin.

Virosomes and immunostimulatory complexes (ISCOM) can also be used to prime CD8+ T cells for responses to protein antigens (Zhou and Huang, 1995). Virosomes are liposomes containing viral glycoproteins and lipids (Almeida et al., 1975) while ISCOM are cage-like structures composed of cholesterol, phosphatidyl choline proteins and Quil A, a partially purified saponin (Villacres et al., 1998). Using ISCOM with biotinylated influenza virus glycoproteins, the intracellular trafficking of ISCOM was studied by electron microscopy. Antigens from ISCOM were detected in the cytosol of peritoneal exudate cells within 5 minutes of exposure (Villacres et al., 1998), indicating that ISCOM rapidly penetrate cells. Despite the relative ease (using fluorescent or fluorescent-labeled proteins), no studies have probed the pathways by which ISCOM enter the cell or which pathways are available to process ISCOM derived antigens. Because the viral glycoproteins mediating cell entry vary depending on virosome construction, these observations are also likely limited to the type of virosome used in the study. Influenza virosomes are internalized by receptor mediated endocytosis (Bron et al., 1994). Fusion with the endosomal compartments is necessary to deliver molecules encapsulated within the virosome to the cytosol (Bron et al., 1994; Bungener et al., 2002). Similar results were observed using Sendai virosomes (Nakanishi et al., 2000). These results imply that virosomes can be used to target proteins to the cytosol, making virosomes a potentially useful for activating T cells *in vivo* and as such a tool for vaccination strategies.

1.3.1.3.4.3 Soluble Antigens

MHC class I processing and presentation of exogenous soluble proteins was described prior to in depth analysis of the mechanisms. Bevan and Carbone found that splenocytes exposed

to soluble ovalbumin *in vitro* were able to prime CD8+ T cells *in vivo* (Carbone and Bevan, 1990). Rock *et al.* then demonstrated that splenocytes, but not a B cell line could present ovalbumin to an ovalbumin-specific T cell hybridoma (Rock et al., 1990). These results implied that pAPC had the capacity to generate determinants from soluble proteins. Norbury *et al.* then demonstrated that macropinocytosis by both macrophages (Norbury et al., 1995) and dendritic cells (Norbury et al., 1997) resulted in the delivery of exogenous proteins to the cytosol. Cytosolic processing of soluble proteins was further implicated by the TAP-dependent presentation of ovalbumin by both DC and macrophages (Brossart and Bevan, 1997). These studies imply that pAPC take up soluble antigens by macropinocytosis and process them in a cytosolic dependent manner. The finding that ovalbumin, a soluble protein, can be cross-presented in this manner is especially important to our studies, which use ovalbumin as a model antigen.

1.3.1.3.4.4 Degradation Intermediates

An *in vitro* study by Serna *et al.* is of particular interest to our examination of the form of antigen transferred in cross-priming *in vivo* (Serna et al., 2003). This study examined the ability of protein degradation intermediates to serve as substrates for cross-presentation *in vitro*. Using T cell hybridoma activation as a readout for cross-presentation, Serna *et al.* demonstrated that whole protein could serve as the form of transferred antigen but protein degradation intermediates were preferred. The system used by Serna *et al.* involved antigen donor cells transfected with plasmids expressing various lengths of ovalbumin under the control of a T7 inducible promoter. T7 was supplied by infection of the cells with a vaccinia virus expressing T7. This elegantly permitted the expression of ovalbumin in the context of infection without the possibility of infection of the APC. Serna *et al.* demonstrated that the hybridoma response to cross-presentation of full length OVA was greatly reduced compared to engineered intermediate-length

proteins containing the ovalbumin determinant. No response was detected to the minimal antigenic peptide, $OVA_{257-264}$, despite the fact that $OVA_{257-264}$ should have access to cytosolic chaperones. A minimal antigenic peptide, $OVA_{257-264}$, targeted to the ER also failed to elicit a T cell response via cross-presentation despite the fact that this ER targeted peptide should be able to interact with the ER resident chaperone gp96, and thus induce cross-presentation. These results indicated that the form of antigen transferred in cross-presentation was likely to be protein, not peptide. To test this hypothesis, cells expressing full length or engineered intermediate length proteins containing $OVA_{257-264}$ were treated with proteasome inhibitors. If the form of antigen for cross-presentation was full-length protein, inhibition of proteasomal degradation would result in the enhancement of cross-presentation as it would increase the pool of intact proteins available to be transferred. However, this was not the case as proteasomal inhibition decreased cross-presentation of both full-length OVA as well as that of the engineered intermediate length proteins containing $OVA_{257-264}$, indicating that proteasomal products, not substrates were the form of antigen transferred in cross-presentation. Several flaws in these experiments confound the interpretation of these results. First, the APC used in these studies were bone-marrow derived macrophages, capable of cross-presentation, but not optimal cross-presenting cells (Mitchell et al., 1998; Norbury et al., 1995; Rock et al., 1990). Second, the interpretation of the proteasome inhibition data is suspect as no controls were shown for the affect of the proteasome inhibitors on the ability of the vaccinia virus to infect the treated cells or synthesize T7. Thus, the decrease in cross-presentation in the presence of proteasome inhibitors may be due to either a decrease in cross-presentation or a decreased level of expression of the OVA proteins due to reduced infection and/or T7 expression (Serna et al., 2003).

***1.3.1.3.5 Nature of Cross-primed Antigen* In Vivo**

In vivo studies using ovalbumin, a soluble protein, suggested that soluble protein injected by itself was not a candidate for the form of antigen transferred as no CD8+ T cell responses were activated (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996). However, ovalbumin could stimulate cross-priming when absorbed onto a microsphere, indicating that particulate or aggregated protein may be a candidate for the form of transferred antigen (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996). Similar results were obtained using the HIV protein gp120 (Shi and Rock, 2002). Ovalbumin could also stimulate cross-priming *in vivo* when it was injected along with, or conjugated to, an adjuvant (Cho et al., 2000; Durand et al., 2004; Sparwasser et al., 2000). Adjuvants were demonstrated to activate APC to express co-stimulatory molecules, suggesting that the activation status of the APC may play a role in the form of antigens which can be cross-primed (Cho et al., 2000; Durand et al., 2004; Sparwasser et al., 2000). Activation of APC in our experiments is taken care of by our use of viruses or virally infected cells.

In vivo experiments by the Srivastava group suggested that heat shock protein (HSP)-bound peptides were the form of antigen transferred. Srivastava *et al*. and Ulrich *et al*. both found that HSP isolated from tumors were immunogenic and stimulated tumor specific CD8+ T cells (Srivastava et al., 1986; Ullrich et al., 1986). HSP isolated from the tumors were later revealed to be HSP90 and gp96, both of which function as molecular chaperones, HSP90 in the cytosol/nucleus and gp96 in the ER (Nicchitta, 1998). It was theorized that similar to HSP70 (Flynn et al., 1989; Greene et al., 1995), gp96 would be able to bind to peptides, essentially hypothesizing that the basis of tumor-specific immunity was the cross-priming of gp96-bound peptides. In support of this hypothesis, it was reported that gp96 was tightly bound in a 1:1 ratio with peptides ranging in size from 400 to 2000 Daltons (Li and Srivastava, 1993). However, subsequent reports have since demonstrated that peptides bound to gp96 were dissimilar in structure to MHC class I binding peptides (Demine and Walden, 2005) and that “purified” gp96

preparations actually contained immunogenically relevant levels of contaminating proteins (~473x10^6 molecules of contaminating protein/μg of gp96) (Reed et al., 2002), suggesting that HSP may actually function more as adjuvants than as the source of antigen transferred in cross-priming *in vivo*.

Nonetheless, the ability of HSP-bound peptides to be presented on MHC class I was examined *in vitro* where it was discovered that HSP-bound peptides, but not free peptides or serum albumin-bound peptides, introduced into the cytosol of antigen presenting cells were efficiently processed and presented on MHC class I (Binder et al., 2001). This study directly introduced HSP-peptide complexes directly into the cytosol; thus it did not address whether HSP-peptide complexes are transferred, but simply that they could be processed and presented on MHC class I. The ability of HSP-bound peptides to serve as the antigenic substrate for cross-priming *in vivo* was then examined by two different groups with contrasting results.

The two groups examining the ability of HSP-bound peptides to serve as the antigenic substrates for cross-priming *in vivo* used similar systems. Shen and Rock immunized mice with antigen-expressing cells or subcellular fractions from antigen-expressing cells while Binder and Srivastava immunized mice with cell lysates from antigen-expressing cells. Both groups depleted intact antigenic protein using antibodies but only Binder and Srivastava depleted HSP and HSP-peptide complexes in this manner. Shen and Rock addressed the ability of HSP to cross prime by expressing different forms of antigen that were targeted to different areas of the cell. In Shen and Rock's experimental design, if HSP-peptide complexes are the source of antigen transferred, then the subcellular location of cross-priming activity should be independent of the subcellular location of the protein antigen. The subcellular location of cross-priming activity will be independent of the subcellular location of the protein antigen because cytosolic and ER HSP will be loaded with peptides generated in the cytosol and transported into the ER due to degradation of the antigenic protein regardless of its subcellular location. One week post-immunization, cross-

priming was measured indirectly by Binder and Srivastava and directly by Shen and Rock. Binder and Srivastava harvested CD11c+ DC from the immunized mice and measured cross-priming by the ability of the harvested DC to stimulate T cell hybridomas *in vitro*. In contrast, Shen and Rock harvested splenocytes from immunized mice, re-stimulated the splenocytes *in vitro* and then performed CTL assays to determine the number of CD8 T cells primed by in the immunized mice. Based on their study, Binder and Srivastava concluded that HSP-peptide complexes were both necessary and sufficient to stimulate cross-priming *in vivo* (Binder and Srivastava, 2005). However, Shen and Rock concluded that cellular protein was the form of antigen transferred during *in vivo* cross-priming (Shen and Rock, 2004). However, the use of cell lysates, subcellular fractions, antibody-mediated depletion and T cell hybridomas confound the interpretation of these results. Immunization with cell lysates or subcellular fractions is likely to be useful therapeutically, but it is unlikely that the immunizing dose of cell lysates or subcellular fractions mimics the amount of lytic cell death induced naturally by viruses and tumors. Thus, it is unlikely that immunization with cell lysates or subcellular fractions is a physiologically relevant manner in which to examine the requirements of cross-priming *in vivo*. Antibody depletion of protein, HSP, or HSP-peptide complexes does not ensure that antigenically relevant amounts of protein, HSP or HSP-peptide complexes are absent from the cell lysates. Antibodies used in antibody depletion may also be unable to recognize protein degradation intermediates due to conformational changes in those proteins induced by degradation. Thus, the conclusion that HSP-peptide complexes or cellular protein is the definitive form of antigen transferred *in vivo* is flawed because the presence of degradation intermediates cannot be ascertained. Furthermore, the use of T cell hybridomas by Binder and Srivastava to detect the cross-priming of cell lysate-associated antigen is a very indirect assay and is not a particular sensitive method of detecting antigen transfer. This is especially true for this study because mice with transgenic T cells

specific for antigens utilized in this study are commercially available (Binder and Srivastava, 2005).

A later study by the Srivastava group attempted to address the flaws of the original study by using different techniques (Binder et al., 2007). Cross-priming was measured by the activation of T cells in immunized mice, a more sensitive measure of cross-priming than the indirect activation of T cell hybridomas. In this study, the Srivastava group used subcellular fractions rather than cell lysate. However, antibodies were still used to identify the contents of the fractions. Thus, this later study still does not address the presence of degradation intermediates within the fractions and thus cannot demonstrate that HSP-complexes are sufficient for cross-priming *in vivo* (Binder et al., 2007).

While these studies demonstrate the controversy regarding the form of antigen transferred to the APC *in vivo*, they do not conclusively demonstrate that either protein or HSP-peptide complexes are the form of antigen transferred *in vivo*. In order to determine the form of antigen transferred in cross-priming, a physiologically relevant *in vivo* system is needed. Ideally, such a system would utilize antigen donor cells that express various lengths of an antigenic protein or an infected antigen donor cell that is unable to directly present the antigen or transmit the infectious agent. This would allow the form of the antigen to be probed in two different, physiologically relevant manners. The first method would demonstrate which form of the protein is the preferred form of antigen transferred *in vivo* while the second method would permit the requirement for proteasomal degradation in the antigen donor cell to be probed. The second method could also be used to examine which other cellular functions, such as secretory function or protein synthesis (as long as the antigen was loaded into the cell), are required in the antigen donor cell in order for cross-priming to occur. We intend to use such systems to gain a better understanding of the requirements of the cross-priming pathway *in vivo*.

1.3.2 MHC class II Antigen Processing and Presentation

1.3.2.1 MHC class II molecules

MHC class II consists of a non-covalent complex of two chains, α and β, that both span the membrane. Both the α and the β chain are encoded within the MHC gene complex and are made up of two domains; α_1 and α_2 for the α chain and β_1 and β_2 for the β chain (Figure 1.5A). α_1 and β_1 domains combine non-covalently to form a peptide binding groove similar to that of MHC class I with α-helices forming the sides of the groove and β-pleated sheets forming the actual groove (Figure 1.5B) (Kaufman et al., 1984). The α_1 domain is relatively invariant, with the allelic variability lying mainly in the β_1 domain, which is also the domain that binds covalently to antigenic peptide (Fremont et al., 1996). The invariant α_2 and β_2 domains share structural similarities with an immunoglobulin constant domain and both span the membrane. Similar to the recognition of MHC class I by CD8, CD4 recognizes MHC class II at the invariant β_2 domain.

Both chains of MHC class II are synthesized in the rough ER and transiently form high molecular weight aggregates containing the immunoglobulin heavy chain binding protein, BiP (Bonnerot et al., 1994; Marks et al., 1995; Roche et al., 1991). These aggregates contain both single chains and MHC class II heterodimers (Bonnerot et al., 1994; Marks et al., 1995). Association with the invariant chain and calnexin releases MHC class II from the aggregates (Anderson and Cresswell, 1994). Calnexin release correlates with the formation of invariant chain: MHC class II nonamers made up of one trimeric invariant chain and three MHC class II heterodimers (Figure 1.6A) (Anderson and Cresswell, 1994; Cresswell, 1994). Invariant chain is a type II glycoprotein that binds the nascent MHC class II across the peptide binding groove. Invariant chain binding by MHC class II is thought to have several functions. First, the binding

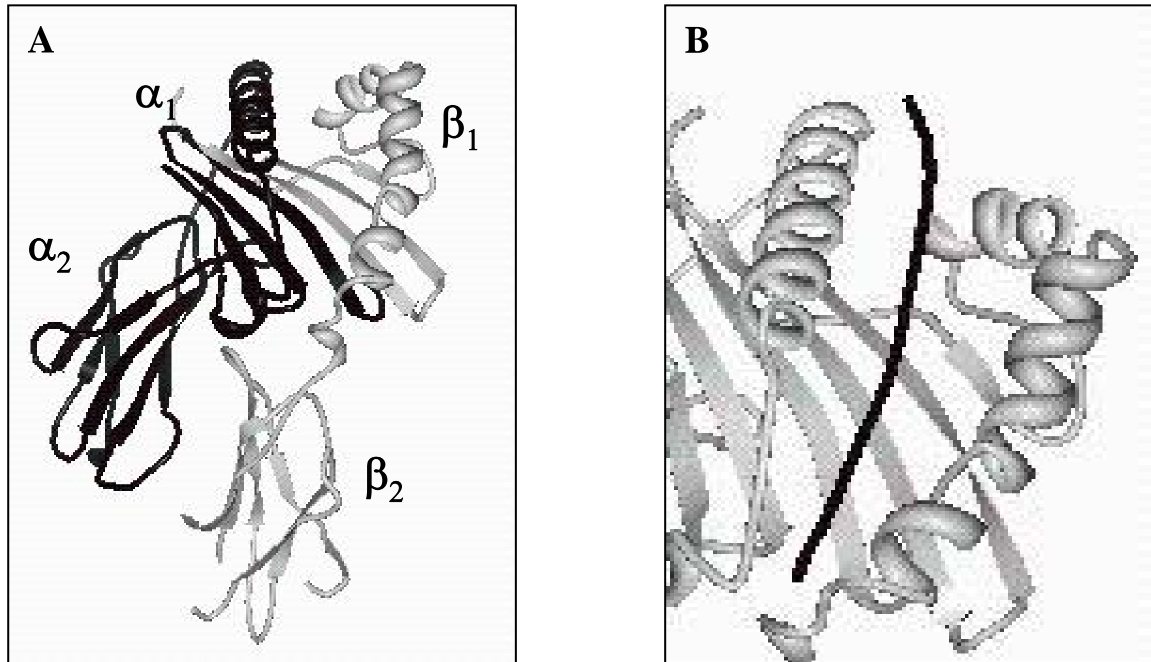

Figure 1.5 Structure of MHC Class II.

(A) MHC Class II molecule with the alpha chains shown in black and beta chains shown in gray. (B) Peptide (black) in an MHC class II (gray) binding groove. Note how the ends of the peptide are permitted to extend out of the binding groove. Figures were generated using data submitted by Wilson et al (Zhu et al., 2003) to the RCSB Protein data bank, PDB number 1MUJ. PDB file was then manipulated using the Protein Workshop on the RCSB Protein Data bank (Moreland et al., 2005).

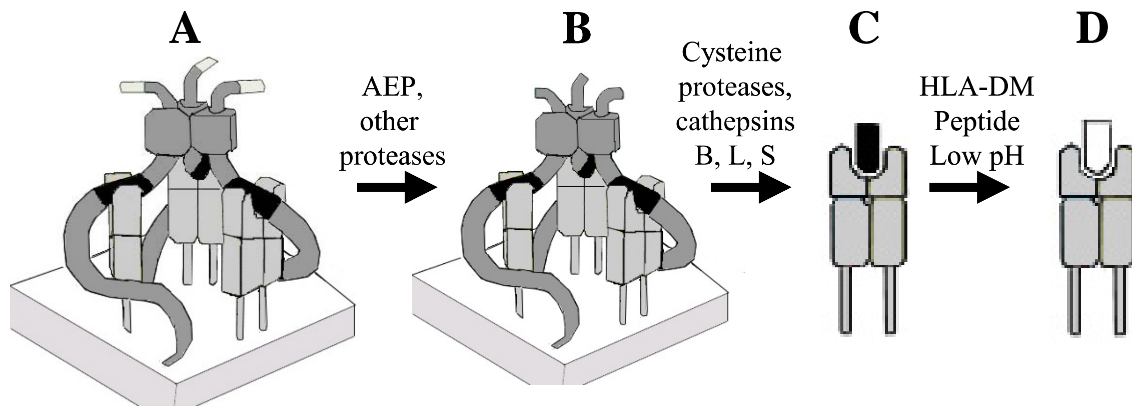

Figure 1.6 Overview of MHC class II loading and maturation.

The invariant chain binds to newly synthesized MHC class II molecules, forming a nonamer (A). In acidified endocytic vesicles, asparginine-specific endopeptidase (AEP) cleaves the C-terminal domain of the invariant chain, leaving a 10kb invariant chain fragment binding the three MHC class II molecules together (B). Further degradation of the invariant chain removes all but the **CL**ass II associated **I**nvariant chain **P**eptide (CLIP) from the MHC class II dimer (C). The class II-like molecule, HLA-DM, binds to MHC class II:CLIP complexes, catalyzing the release of CLIP and the binding of antigenic peptides (D).

of invariant chain to MHC class II heterodimers prevents the heterodimers from forming aggregates. This was demonstrated by the accumulation of aggregates in the ER of cells lacking invariant chain expression (Bonnerot et al., 1994; Marks et al., 1995). Second, by binding to MHC class II across the peptide groove, invariant chain prevents MHC class II from binding to the large quantities of unfolded proteins present in the ER (Roche and Cresswell, 1990; Teyton et al., 1990). The region of the invariant chain critical for interacting with MHC class II (Bijlmakers et al., 1994; Freisewinkel et al., 1993; Romagnoli and Germain, 1994) was demonstrated to bind MHC class II even when cleaved to a peptide (Ghosh et al., 1995), causing it to be named CLIP for **CL**ass II associated **I**nvariant chain **P**eptide. CLIP was demonstrated to prevent binding of synthetically labeled peptides *in vitro* (Roche and Cresswell, 1990; Teyton et al., 1990) and the absence of invariant chain (and thus CLIP) resulted in large ER polypeptides being presented on the cell surface (Busch et al., 1996). Finally, the last function of the invariant chain is to ensure transport of MHC class II molecules through the Golgi to the vesicular compartments, where the invariant chain can be exchanged for peptide (Bikoff et al., 1993; Elliott et al., 1994; Viville et al., 1993). Transport from the ER through the Golgi to the endosomes is mediated by two endosomal-targeting sequences in the cytoplasmic tail of the invariant chain (Motta et al., 1995; Odorizzi et al., 1994; Pieters et al., 1993).

Arrival of the invariant chain:MHC class II nonamer in the proper endosomal compartment results in the sequential degradation of the invariant chain initiating at the C-terminus due to the low pH and proteases found in the endocytic pathway (Blum and Cresswell, 1988; Nowell and Quaranta, 1985). It is not clear where these degradation events occur as there is evidence of invariant chain degradation throughout the endocytic pathway. The proteases used to degrade the invariant chain are redundant; knocking out one protease does not affect the degradation of the invariant chain. This redundancy may be due to the large number of endosomal compartments to which the invariant chain:MHC class II complex have access.

Degradation of the invariant chain begins with a C-terminal cleavage that leaves a 10kb amino-terminal domain called p10 bound to MHC class II (Figure 1.6B). This cleavage is mediated by asparginine-specific endopeptidase (AEP), although other proteases can cleave p10 as well (Manoury et al., 2003). The degradation of the invariant chain then proceeds to the CLIP fragment with degradation by cathepsins (Figure 1.6C). Cathepsin L has been demonstrated to degrade the invariant chain in the thymus (Nakagawa et al., 1998) while cathepsin S is important in the degradation of the invariant chain in both B cells and dendritic cells (Driessen et al., 1999; Nakagawa et al., 1999). Invariant chain degradation in macrophages deficient in cathepsin L, cathepsin S or both was unaffected (Shi et al., 2000), suggesting that other proteases also play a role. Other studies demonstrated that deficiencies in the degradation of the invariant chain can be MHC class II molecule specific in cathepsin knock out mice (Honey and Rudensky, 2003), suggesting that MHC class II molecules traffic to different endosomal compartments. Together, these studies suggest that it is important that an antigen be targeted to the same endosomal compartment as the MHC class II molecule capable of binding it. Removal of the final fragment of the invariant chain, CLIP, is involved with peptide loading onto MHC class II and will be discussed below as will MHC class II trafficking to the cell surface (Figure 1.6D).

1.3.2.2 Classical MHC II Processing Pathway

The classical MHC class II processing pathway involves the processing of exogenous proteins (Figure 1.7). MHC class II is expressed chiefly on pAPC, although other cells can express MHC class II under conditions of inflammation (Cella et al., 1997; Inaba et al., 2000; Pierre et al., 1997; Turley et al., 2000). pAPC are extremely effective at importing proteins from their microenvironments, allowing presentation of exogenous proteins on MHC class II. Activation of the innate immune response produces inflammatory conditions which signal pAPC

to more efficiently process and present imported proteins. Recognition of MHC class II by CD4+ T cells activates CD4+ T cells to secrete cytokines and express co-stimulatory molecules. These cytokines and co-stimulatory molecules are responsible for enhancing the humoral response as well as generating immunological memory.

1.3.2.2.1 Properties of MHC II binding peptides

Unlike MHC class I, the peptide binding groove of MHC class II is open at both ends, which permits the binding of longer peptides (Figure 1.5). Peptides that bind to MHC class II are at least 13 amino acids long, but can be much longer (Falk et al., 1994; Rudensky et al., 1992; Rudensky et al., 1991). The groove anchored peptide stretch is generally 9 amino acids long with residues 1, 4, 6 and 9 of this stretch held in binding pockets within the groove (Stern et al., 1994). The binding preferences of each positional pocket depend on the particular MHC class II molecule. The peptide stretch bound by the groove of MHC class II can appear at any point within the peptide sequence, confounding efforts to predict MHC class II peptides. The amino terminal and carboxy terminal residues of this stretch hang out of the groove. Interactions between the side-chains of these residues and the side chains of the amino acids lining the groove of MHC class II appear to stabilize the binding (reviewed in (Rammensee, 1995)). Thus, our studies examining the MHC class II processing pathway will not use minimal antigenic peptide.

1.3.2.2.2 Origins of Proteins for Presentation on MHC class II

The classical MHC class II processing pathway processes and presents exogenous proteins. Because these proteins are processed in the endocytic compartments of the cell, cellular proteins present in these compartments are also available to the MHC class II processing pathways. Acquisition of exogenous antigen requires endocytosis, the process by which the cell internalizes exogenous proteins. The three routes of endocytosis used for acquiring proteins for

MHC class II presentation are phagocytosis, macropinocytosis and clathrin-mediated endocytosis (Figure 1.7).

1.3.2.2.3 Processing and Loading Peptides onto MHC class II

It is currently unclear whether peptides are processed first and then loaded onto MHC class II or whether unfolded but intact antigen is loaded onto MHC class II and then processed. While text books and diagrams nearly all depict the first scenario of MHC class II processing (Figure 1.8 peptide capture) (Janeway, 2005; Paul, 2003), the first scenario does not explain how peptides of the proper length accumulate in endocytic compartments long enough to be loaded onto MHC class II. Generation and accumulation of proper length peptides for loading onto MHC class II is better addressed by the second scenario (Figure 1.8 epitope capture). The open ends of the MHC class II peptide binding groove permit MHC class II to bind unfolded but intact antigen (Castellino et al., 1998; Donermeyer and Allen, 1989; Sette et al., 1989); rescuing the antigen from degradation in endocytic compartments (Deng et al., 1993; Sercarz and Maverakis, 2003; Werdelin, 1986). The protruding ends could then be trimmed by endo- and exo-peptidases whilst the peptide in the MHC class II binding groove is protected from degradation (Donermeyer and Allen, 1989; Mouritsen et al., 1992). Evidence for the epitope capture pathway was shown by studies where MHC class II co-precipitated with long poly-peptides in cells where the lysosomes were inactivated by inhibitors of acidification (Castellino et al., 1998; Villadangos et al., 2000). Whether these long polypeptides were then trimmed into immunogenic peptides remains unknown.

Despite the evidence for long-polypeptides binding to MHC class II before being trimmed, there is evidence that some proteins must be processed before binding to MHC class II. C57BL/6 mice, which lack an enzyme that cleaves the C-terminal arginine on the MHC class II epitope from hen egg lysosome (HEL), are incapable of presenting HEL on MHC class II.

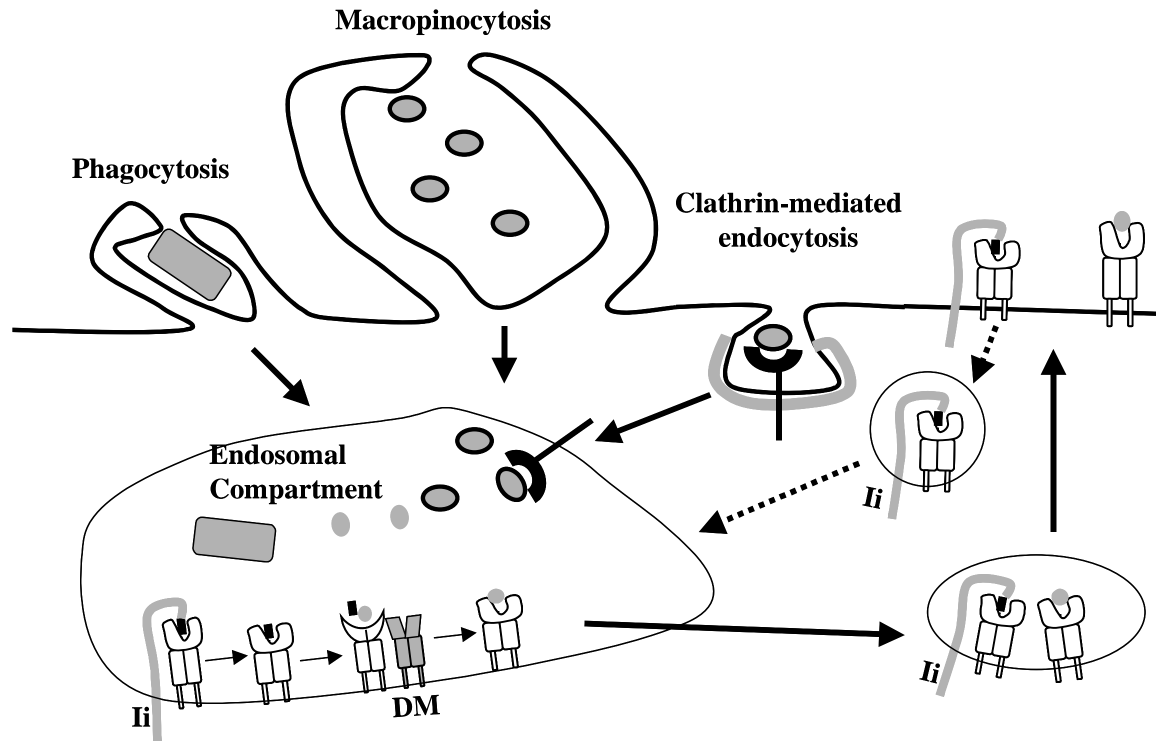

Figure 1.7 Overview of how exogenous antigens are taken up and presented by MHC class II.

Exogenous antigens are taken up via phagocytosis, macropinocytosis or clathrin-mediated endocytosis. The antigens are then transported through the endocytic pathway until they reach the endolysosomal compartment where peptide loading takes place. The endolysosomal compartment where peptide loading takes place is a vesicle with the properties of both and endosome and a lysosome. This endolysosomal compartment is identified by the presence of MHC class II; invariant chain (Ii); molecules involved in loading and editing peptide binding, such as DM; and peptides available for loading onto MHC class II. Once MHC class II reaches this compartment, the invariant chain is degraded, leaving CLIP to block the peptide binding groove. Interaction with DM changes the conformation of the MHC class II peptide binding groove, releasing CLIP and allowing a peptide to bind to MHC class II. The MHC class II-peptide complex is then transported to the cell surface via tubules. The dashed lines indicate one of the many paths MHC class II can take to reach the endolysosomal compartment where peptide loading occurs.

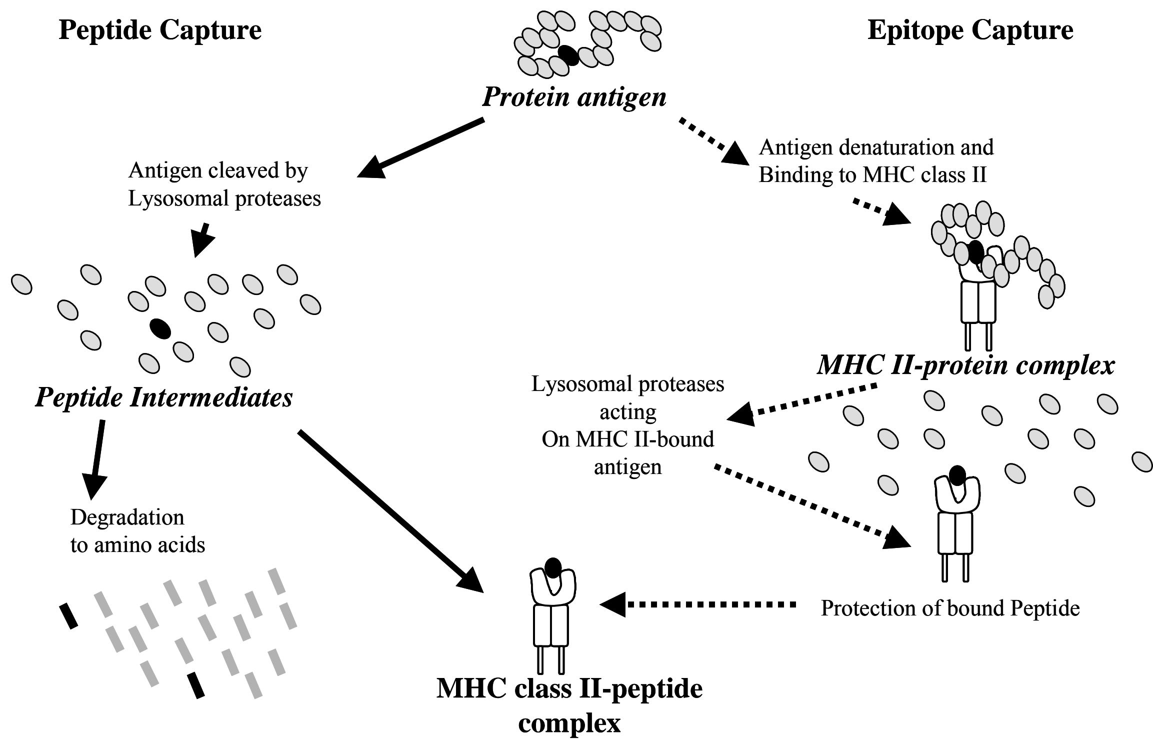

Figure 1.8 Possible pathways for generation of peptide-MHC class II-complexes.

The "Peptide Capture" pathway (solid line) is the pathway most commonly depicted for the generation of peptide-MHC class II-complexes. In this pathway protein antigens are first cleaved by lysosomal proteases, and the resulting peptides then bind to MHC-II. In the "Epitope Capture" pathway, antigens would bind to MHC-II first, and the protruding ends unprotected by MHC-II would be subsequently trimmed by lysosomal proteases. These two pathways are not necessarily mutually exclusive, and antigens can follow a combination of both pathways. Adapted from (Trombetta and Mellman, 2005).

Competitive inhibition assays demonstrated that the presence of the C-terminal arginine in the HEL epitope prevented MHC class II binding; implying that HEL must be processed to remove the arginine prior to being loaded onto MHC class II (Grewal et al., 1995). Together with the evidence for protein binding to MHC class II prior to processing, this evidence implies that protein processing and antigen loading in the MHC class II pathway may be antigen-dependent.

Regardless of the order of antigen processing and loading onto MHC class II, processing of antigen for presentation on MHC class II begins with the unfolding of the protein. Protein unfolding occurs in two manners. First, acidic pH reduces the number of inter- and intra-molecular disulfide bonds (Collins et al., 1991). Second, the γ-IFN-inducible lysosomal thiol reductase (GILT) catalyzes the removal of disulfide bonds at low pH further unfolding the protein (Arunachalam et al., 2000; Phan et al., 2000). A single cleavage event may be required in order to "unlock" the unfolded protein and make it subject to further degradation for presentation. The concept of "unlocking" an antigen for degradation was hypothesized because blocking the action of specific endopeptidases blocks presentation of epitopes found within the protein. For example, blocking the action of asparginine-specific endopeptidase (AEP) by blocking its cleavage site prevented presentation of tetanus toxin epitopes (Antoniou et al., 2000; Manoury et al., 1998).

Once the antigenic protein is "unlocked" further degradation can occur. This degradation can be mediated by proteases such as cathepsins. A great deal of redundancy exists within these degradation pathways. Knocking out one cathepsin may or may not have an effect on antigen presentation, as the requirement for each cathepsin can be cell type or antigen specific. Degradation of proteins by cathepsins is highly regulated. Cathepsins are produced as pro-enzymes that require cleavage in order to be activated. Cystatins, cathepsin inhibitors, can also bind to and inactivate cathepsins. Cytokine signaling can both up-regulate and down-regulate degradation. Inflammatory cytokines such as IFN-γ, TNF-α or IL-6 act to increase presentation on MHC class II by activating transcription of cathepsins and maturation from the pro-enzyme to

the mature form (IFN-γ) (Lennon-Dumenil et al., 2001) or by lowering the pH of the vesicle (TNF-α and IL-6) to increase the unfolding of the antigenic proteins and enhance activation of proteases (Drakesmith et al., 1998; Fiebiger et al., 2001). IL-10, however, down-regulates degradation of antigen and MHC class II processing by increasing the pH of the endocytic compartments and inhibiting protease and hydrolase activity (Fiebiger et al., 2001).

Loading of peptides onto MHC class II requires that the invariant chain first be degraded. Removal of the final fragment of the invariant chain, CLIP, is mediated by a MHC class II-like chaperone, DM (Fling et al., 1994; Morris et al., 1994). DM is expressed from the MHC gene complex, where it is encoded in close proximity to the other antigen processing genes. DM is expressed as a heterodimer of α and β chains, similar to MHC class II. Unlike MHC class II, DM does not require peptide binding for stabilization. DM is found throughout the endocytic pathway, but is concentrated in the endolysosomal compartment where peptides are loaded onto MHC class II (Figure 1.7). DM binds to the MHC class II:CLIP complex, altering the conformation of MHC class II so that CLIP is released, leaving the peptide binding groove open. DM remains bound to MHC class II until a peptide is bound. However, DM will attempt to rebind MHC class II complexes, a process which allows peptides with weak affinity for the MHC class II binding groove to be released. This process continues until MHC class II binds a peptide of sufficient affinity (Denzin et al., 1996; Jensen et al., 1999; Kropshofer et al., 1996).

Peptide binding to MHC class II is also regulated by another MHC class II-like molecule DO. DO, like MHC class II and DM, is expressed from the MHC gene complex and is composed of an α and β chain. DO is a negative regulator of DM activity that binds to DM and prevents DM from interacting with MHC class II. While inflammatory signals up-regulate DM expression, they have no effect of DO, which remains at homeostatic levels (Alfonso et al., 1999;

Denzin et al., 1997; van Ham et al., 1997). Thus, inflammatory signals increase presentation on MHC class II by permitting more DM molecules to interact with MHC class II.

Peptides also bind to MHC class II molecules that are recycled from the cell surface. Roche *et al.* first characterized nascent MHC class II molecules being recycled from the cell surface to the endosomes (Roche et al., 1993). Nascent MHC class II molecules were identified by the fact that they were still bound to invariant chain. Two distinct pathways of antigen presentation on MHC class II molecules was then identified using inactivated influenza virus (Pinet et al., 1994). One pathway utilized the "classical" MHC class II processing pathway and required newly synthesized MHC class II and the function of DM. The alternate pathway did not require protein synthesis of MHC class II and also did not require DM. In a separate study Pinet *et al.* then demonstrated that the antigen using the alternative pathway bound to recycled MHC class II (Pinet et al., 1995). The kinetics of loading antigen on recycled MHC class II were more rapid than those of the classical pathway, suggesting that the alternative pathway makes use of antigens that would be rapidly degraded and thus unavailable to the classical pathway (Pinet and Long, 1998). Loading of peptide onto recycled MHC class II was further characterized by demonstrating that peptide loading takes place in an earlier endosomal compartment than the ones typically used by newly synthesized MHC class II (Pinet and Long, 1998). While some processing of the antigen was demonstrated to be necessary by Pinet *et al.*, other groups demonstrated that processing was unnecessary as native and partially folded full-length proteins could be presented on recycled MHC class II molecules. (Griffin et al., 1997; Lindner and Unanue, 1996; Vergelli et al., 1997; Zhong et al., 1997). Together, these discoveries imply that a different subset of proteins may be available for loading on recycled MHC class II. To enhance presentation on MHC class II with rational vaccine design, it may be beneficial to take advantage of both the nascent and recycled MHC class II molecules.

1.3.2.3 Non-Classical MHC Class II Antigen Processing Pathway

Although MHC class II molecules avoid binding to endogenous proteins in the ER by binding to the invariant chain, many peptides derived from endogenous proteins can be eluted from cell-surface MHC class II molecules (Mouritsen et al., 1994; Newcomb and Cresswell, 1993; Rudensky et al., 1991). Biochemical studies revealed that the majority of the peptides bound to MHC class II are derived from endogenous proteins normally found on the cell surface, endocytic or secretory compartments (Rudensky et al., 1991). Cytosolic antigen-derived peptides were also eluted from MHC class II molecules (Chicz et al., 1994; Chicz et al., 1993; Rudensky et al., 1991). Theoretically, endogenous proteins could be taken up from apoptotic or necrotic cells. However there is no evidence for the presentation of endogenous proteins on MHC class II via this route, probably because it is so difficult to distinguish endogenous protein that enter the cell through endocytosis from endogenous proteins. Endogenous proteins do access the endosomal degradation pathway through autophagy. Autophagy is a process that literally means "self-eating." Autophagy is normally triggered by cell stress, although there is evidence that autophagy occurs under normal, basal cell conditions (Schmid et al., 2007). The study that provided evidence for autophagy under basal conditions used an engineered antigen fused with a protein involved with the formation of autophagosomes. Thus, the fusion protein may actually trigger the autophagy observed in this study. In autophagy, a double-membraned vesicle called an autophagosome forms and subsequently fuses with the endolysosome (Yorimitsu and Klionsky, 2005). The resulting single membrane structure is called the autophagic body. The membrane of the autophagic body is subsequently dissolved by lysosomal esterases, releasing the cytoplasmic contents of the autophagic body for degradation by lysosomal proteases (Yorimitsu and Klionsky, 2005). Clearly, MHC class II molecules which traffic throughout the endocytic pathway would have access to peptides generated by lysosomal degradation of the cytosolic

proteins. Recent studies implicate autophagy in the processing of endogenous antigens for MHC class II presentation. Metabolically stressed EBV-transformed cells up-regulated presentation of cytosolic and nuclear proteins on MHC class II (Dengjel et al., 2005). The metabolic stress-induced autophagy in these cells promoted peptide presentation on MHC class II molecules by decreasing active cathepsins in the endocytic compartments (Dengjel et al., 2005). MHC class II presentation of peptides from autophagic degradation has been reported for several antigens (Brazil et al., 1997; Nimmerjahn et al., 2003; Paludan et al., 2005; Schmid et al., 2007). Of particular interest to our studies using multiple viruses to express the same antigen, the presentation of an endogenously synthesized viral protein has already been demonstrated on MHC class II molecules after induction of macroautophagy (Paludan et al., 2005).

Autophagy may be the mechanism by which endogenous antigens are delivered to endosomal compartments for presentation on MHC class II, but how and where are the endogenous antigens processed to peptides? Hypothetically, autophagy could deliver intact proteins or degradation intermediates to the endolysosome. Jaraquemada *et al.* reported that expression of influenza virus matrix protein in APC resulted in presentation on MHC class II. This presentation was abrogated when chloroquine was used to inhibit lysosomal acidification, implying that the matrix protein was degraded in the lysosomal compartment (Jaraquemada et al., 1990). Other groups agreed with the findings of Jaraquemada while also demonstrating that proteasome inhibition enhanced presentation (Gueguen and Long, 1996; Nimmerjahn et al., 2003), presumably by extending the life of endogenous proteins until they could be transferred to the endolysosome by an autophagosome. Subsequent studies, however, demonstrated the requirement for cytosolic processing of endogenous proteins presented on MHC class II. Inhibition of cytosolic proteases, such as calpain (calpeptin) and tripeptidyl-peptidase II (TPPII), and proteasomal activity were shown to abrogate the presentation of cytosolic protein on MHC class II (Lich et al., 2000; Mukherjee et al., 2001). Cytosolic processing of endogenous proteins

requires that there be a mechanism by which the generated peptides are loaded onto MHC class II. Several groups have reported a requirement for TAP in the transport of cytosolic-processed endogenous MHC class II peptides (Carmichael et al., 1996; Malnati et al., 1992; Tewari et al., 2005) while others have demonstrated that TAP is not necessary (Malnati et al., 1993; Zhou and Blum, 2004), suggesting that there are multiple mechanisms by which endogenous proteins are processed for presentation on MHC class II.

Clearly more research is needed into the non-classical pathways of MHC class II processing and presentation. While potential pathways and mechanisms have been proposed through *in vitro* studies, little is known about the importance of such pathways on presentation *in vivo*. Our studies using different viruses to express the same antigen will hopefully allow us to clarify the importance of these pathways *in vivo*.

1.4 Need for a Better T Cell Vaccine

At this time, the majority of our successful vaccines are empirically derived (Plotkin, 2005; Pulendran and Ahmed, 2006; Rappuoli, 2004). That is, it is known that these vaccines produce a protective response, generally measured by production of antibodies. The majority of the infectious diseases that still present a problem for humans today are resistant to traditional, empirically derived vaccines. These diseases include those caused by bacteria (tuberculosis), viruses (HIV and influenza) and parasites (malaria). Because empirically derived vaccines typically generate neutralizing antibody responses, it is hoped that vaccines that generate a cytotoxic T cell response will be more successful at inducing immunity to pathogens in which traditional vaccination strategies have failed. To ensure long-term immunity, a T cell vaccine must also activate helper (CD4) T cells. At this time, it has proven difficult to develop a vaccine

that elicits T cell responses. A better understanding of how naïve T cells are primed would aid in the rationale design of vaccines to stimulate cytotoxic and helper T cell responses.

Successful vaccines exploit the memory function of the immune system by exposing the body to a pathogen (or subunit of a pathogen) in order to stimulate long-term protective immunity to the pathogen. Effective vaccines must address two main criteria: effective induction of the immune response and safety. While induction of the immune response may seem obvious, effective vaccines need to induce an appropriate immune response that leads to immunity to infection by a specific pathogen. For example, antibody responses to HIV and dengue virus actually enhance their ability to infect cells *in vitro* (Halstead and O'Rourke, 1977; Halstead et al., 1980; Hotta et al., 1984; Matsuda et al., 1989; Takeda et al., 1988). *In vivo* evidence also shows that antibodies specific for dengue virus enhance infection *in vivo* (Kliks et al., 1988; Kliks et al., 1989; Takada and Kawaoka, 2003). Thus strategies for HIV and dengue virus vaccine design need to consider that invoking an antibody response may not lead to immunity but enhanced pathology. Safety refers to the fact that in order to be effective vaccines must induce an encounter with the pathogen without causing the pathogenic events associated with the virulent pathogen or other deleterious side effects.

Live attenuated virus vaccines and recombinant viral vaccines produce a strong humoral and T cell responses, generally with a single immunization (Jiang et al., 2006; Kennedy et al., 2004; Kumar and Seth, 2004; Lorin et al., 2004; Zhan et al., 2007). Thus, viruses that cause mild to no disease in humans have been proposed as vectors to stimulate T cell immunity to pathogenic viruses such as HIV and influenza A virus. To be considered as a good candidate for use as a viral vaccine vector, the ideal vector needs to: grow to high titer in culture, be able to accommodate large transgenes, have a low prevalence of existing immunity, and be able to replicate within the immunized host without causing serious disease. Because viral vectors are proposed as a method for activating strong T cell responses, we decided to examine how the viral

vector affects the antigen processing and presentation pathways available to a specific antigen. The viruses used in our experiments are detailed below.

1.5 Viruses Used in Our Experiments

We chose to use both Vesicular Stomatitis Virus (VSV) and Vaccinia Virus (VV) in our studies because they are both strong candidates for vaccine vectors. VSV and VV grow to high titers and are capable of accepting large transgene sequences. Several studies have demonstrated that VSV expression of the hemagglutinin protein from influenza resulted in protective immunity to influenza, even when VSV was attenuated by deletion of the gene for the VSV-G protein (Roberts et al., 1999; Roberts et al., 1998). Vaccinia virus, a vaccine strain of smallpox derived from cowpox, was the first recombinant vector used to elicit cytotoxic T cell responses to transgenes (Bennink and Yewdell, 1990; Panicali et al., 1983). Experiments using VV as a vector have revealed that the life cycle of the virus is able to effect the expression, processing and presentation of the transgene. The promoter under which the transgene is expressed can determine how much of the transgene is expressed; yet the higher level of transgene expression observed from the VV late promoter does not lead to a more robust T cell response but to a feeble one (Coupar et al., 1986). This was later correlated to be due to an abortive infection of cultured dendritic cells by vaccinia virus in which late genes were poorly expressed (Bronte et al., 1997). These observations emphasize the importance of studies to determine how expression, processing and presentation of the transgene will be affected by the viral life cycle. The life cycles and immune response stimulated in mice by our two chosen viruses will be discussed below.

1.5.1 Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) is an enveloped Rhabdovirus capable of producing infections in many species and occasionally in humans (Fields and Hawkins, 1967). Laboratory strains of VSV can be divided into two antigenically distinct species or serotypes called VSV-Indiana and VSV-New Jersey, the main difference between the two serotypes is in the glycoprotein (Kelley et al., 1972). VSV is a non-segmented negative strand RNA virus of approximately 11.2kb that contains 5 genes in the order of 3'- N-P-M-G-L-5' (Rose, 1987). VSV particles are bullet shaped and contain a nucleocapsid consisting of single-stranded RNA tightly encased (Grigera et al., 1992) in the major nucleocapsid protein N and the minor polymerase proteins L and P, and a bilayer membrane associated with the glycoprotein (G) and the matrix protein (M) (Dalton and Rose, 2001). VSV enters the cell by binding to the cell with its glycoprotein (G) spike (Black and Lyles, 1992). VSV does not have a known receptor for viral attachment to the cell; however liposomes containing phosphatidylserine were able to block 80-90% of VSV attachment to Vero cells, indicating that phosphatidylserine may play a role in VSV entry into the Vero cells (Schlegel et al., 1983). VSV enters the cell through endocytosis, and the subsequent reduction of pH in the endosomes triggers a membrane fusion reaction that releases the nucleocapsid core into the cytoplasm (Matlin et al., 1982). Once the nucleocapsid is released into the cytoplasm, the RNA-directed RNA polymerases (L and P) carried by the virion transcribe the negative-strand genome into mRNAs (Baltimore et al., 1970; Emerson and Yu, 1975). Transcription begins at the 3' end of the genome and produces a lead sequence followed by sequential synthesis of the mRNAs encoding the N, P, M, G and L proteins (Abraham and Banerjee, 1976; Ball and White, 1976). The transcriptase pauses, and is attenuated by 20-30% at each of the gene junctions, resulting in a gradient of mRNA production which follows the gene order N>P>M>G>L (Banerjee and Barik, 1992; Iverson and Rose, 1981). During transcription,

the VSV polymerases respond to termination signals, allowing for the generation of discrete leader sequence and mRNAs for each of the proteins. In order to replicate the viral genome, however, the VSV polymerases must ignore these signals in order to replicate the entire genomic strand of RNA. Thus, VSV replication requires a complex of N and P in a 1:1 ration (La Ferla and Peluso, 1989). This complex favors replication over transcription because newly synthesized N selectively encapsidates the leader mRNA (Blumberg et al., 1983) and prevents recognition of the termination signals by P (Banerjee and Barik, 1992). The genome-length plus strand is concomitantly encapsidated by the newly synthesized N protein. The plus-strand RNP then serves as the template for the synthesis of minus-strand progeny, although the requirements for minus strand synthesis has not yet been elucidated (Condra and Lazzarini, 1980; Ghosh and Ghosh, 1982; Hill et al., 1981; Peluso and Moyer, 1983). Newly synthesized N, P and L proteins associate in the cytoplasm with the newly replicated minus-strands to form ribonucleoprotein cores. M protein then binds to and condenses the cores to form a tightly coiled structure (Barge et al., 1993; Newcomb et al., 1982). The M protein is also thought to recruit the cores to the plasma membrane in preparation for viral budding (Chong and Rose, 1993; Chong and Rose, 1994; Li et al., 1993). Once the nucleocapsids are recruited to membrane areas rich in both M and G, interactions between the cytoplasmic tail of G (Metsikko and Simons, 1986; Whitt et al., 1989) or between the membrane bound nucleocapsid-M complexes (Li et al., 1993) presumably drive envelopment of the nucleocapsid cores and budding. Both infectious virions and non-infectious defective interfering (DI) particles are released from infected cells. The number of DI particles released from an infected cell is proportional to the multiplicity of infection (Cave et al., 1985). DI particles contain the same complement of proteins and lipids as infectious particles, however, they are not infectious because 50-80% of the RNA genome is missing (Holland, 1987).

VSV infection has an intense cytopathic effect on infected tissue culture cells. Cellular RNA and DNA synthesis are both inhibited in the same fashion by VSV infection. Only the

leader sequence of the plus-stranded VSV genome was thought to be essential for inhibition of host cell RNA and DNA synthesis (Grinnell and Wagner, 1983; Grinnell and Wagner, 1984; Grinnell and Wagner, 1985; Kinchington et al., 1986; Remenick et al., 1988; Wagner RR, 1984; Weck and Wagner, 1979; Wu and Lucas-Lenard, 1980). However, VSV proteins were also shown to be involved when Poirot *et al.* established that protein synthesis of VSV proteins was required for inhibition of RNA synthesis (Poirot et al., 1985). The matrix protein, M, was demonstrated to possess a transcription-inhibition function separate from that of the leader sequence (Black and Lyles, 1992; Black et al., 1993; Lyles et al., 1988). As a result of both the inhibition of cellular RNA and DNA transcription, cellular protein synthesis is also inhibited by VSV infection (Marcovistz et al., 1983).

1.5.2 Immune Response to Vesicular Stomatitis Virus

As our studies using VSV will be conducted *in vivo*, discussion of the immune response to VSV will be limited to *in vivo* studies. Innate and humoral immunity are essential for controlling the initial VSV infection, but T cell mediated immunity is essential for both viral clearance and long-term defense against re-infection. Each type of immunity to VSV will be discussed below.

1.5.2.1 Innate Immunity to VSV

VSV infection is extremely sensitive to interferons (IFN), particularly interferon-α (IFN-α), (Baxt et al., 1977; Belkowski and Sen, 1987; de Ferra and Baglioni, 1981; Manders et al., 1972; Marcus et al., 1971; Marcus and Sekellick, 1978; Simili et al., 1980) a type I IFN secreted from leukocytes and fibroblasts. Type I IFN offer resistance to virus infection, stimulate the

expression of MHC class I molecules on the surface of the cell and inhibit cellular and viral proliferation (Biron, 1998). Type I IFN also help T cell responses and enhance cross-priming (Ahonen et al., 2004; Beignon et al., 2003; Cho et al., 2002; Lapenta et al., 2006), a facet of the innate immune response which may play an important role in the antigen processing pathways available to an antigen expressed by VSV. IFN-α, affects VSV infection by inhibiting the VSV transcription process. IFN-α treatment of infected tissue culture cells reduces the titer of harvested VSV from $1x10^8$ PFU/mL to less than 1000 PFU/mL (Stojdl et al., 2000). The importance of type I IFN in the primary response to VSV *in vivo* is emphasized by the observation that mice that are defective in type I IFN signaling are susceptible to lethal challenge with VSV (Durbin et al., 1996; Meraz et al., 1996; Muller et al., 1994). It has also been demonstrated that plasmacytoid dendritic cells (DC) are the main cell type producing IFN-α in VSV infection *in vivo* (Barchet et al., 2002), most-likely due to toll-like receptor activation (Liu, 2005).

Toll-like receptors (TLR) are also involved in the innate immune response to VSV infection. TLR are pattern recognition receptors that are required for cellular responses to some viruses and can trigger antiviral signaling pathways leading to IFN responses and maturation of dendritic cells (Bowie and Haga, 2005; Vaidya and Cheng, 2003). Both TLR-7 and TLR-9 recognize single stranded RNA and are expressed on plasmacytoid DC (Liu, 2005; Lund et al., 2004). However, only TLR-7, and not TLR-9, signals plasmacytoid DC to produce IFN-α in response to VSV infection (Diebold et al., 2004; Lund et al., 2004). TLR-9 agonists have been demonstrated to effectively cross-prime CD8 T cells (Zaks et al., 2006), implying that our model antigen expressed by VSV should be able to access the cross-priming pathway. Additionally, there is evidence that TLR-4 ligation by VSV can induce IFN-α production in cultured macrophages (Jiang et al., 2005), although the requirement for TLR-4 signaling in an *in vivo* VSV

infection has not yet been established. Indeed, it is not known how VSV ligates TLR-4, which is known to be responsive to lipopolysaccharides (Nagai et al., 2002; Poltorak et al., 1998).

1.5.2.2 Humoral Immunity to VSV

Humoral immunity is required for control of VSV infection. B-cell deficient mice are unable to control VSV infection and die 5-7 days post-infection (Brundler et al., 1996; Thomsen et al., 1997). Because VSV is a highly cytopathic virus, it is necessary that VSV infection and dissemination be limited as quickly as possible. Like most highly cytopathic viruses, a number of low titer antibodies that recognize VSV are present in the blood of naïve individuals (Hangartner et al., 2006; Haury et al., 1997); some of which are also neutralizing antibodies (Hangartner et al., 2003; Martinez et al., 2004; Ochsenbein et al., 1999). Neutralizing antibodies have a relatively high affinity or avidity for exposed surfaces of virus (Bachmann et al., 1997; Roost et al., 1995) and interfere with the receptor binding and cell entry of viruses either by preventing receptor binding or by preventing the conformational changes in the viral proteins necessary for cellular entry (Schibli and Weissenhorn, 2004; Skehel and Wiley, 2000). VSV neutralizing antibodies are targeted towards the G protein (Kelley et al., 1972; Lefrancios and Lyles, 1982; Luo et al., 1988), which is required for cell entry. Transferred neutralizing antibodies are sufficient to protect mice from lethal challenge with VSV (Lefrancois, 1984). Initial production of VSV IgM neutralizing antibodies is independent of T cell help (Bachmann et al., 1995; Charan and Zinkernagel, 1986; Thomsen et al., 1997), which is thought to be due to cross-linking of the B cell receptor by the densely packed G protein on the viral envelope of VSV (Bachmann et al., 1995). Subsequent class switching to produce neutralizing IgG antibodies, however, is T cell help dependent (Leist et al., 1987). Neutralizing antibodies will be used in our experiments to prevent transfer of

infectious VSV from our infected antigen donor cells to the antigen presenting cells of the host they are injected into.

1.5.2.3 CD4+ T cell Response to VSV

As mentioned above, antibody class switching from IgM to IgG in VSV infection requires CD4+ T cell help as depletion of CD4+ T cells resulted in no detectable VSV-specific IgG (Leist et al., 1987). An adoptive transfer of CD4+ T cells from VSV infected mice into T cell deficient mice was sufficient to prevent T cell deficient mice from succumbing to a subsequent lethal VSV challenge (Thomsen et al., 1997). VSV-specific CD4+ T helper cells are stimulated by antigenic determinants derived from the glycoprotein (G), the nucleoprotein (N) and the matrix protein (M), but only the nucleoprotein (N) is able to trigger an IgG response to a different serotype of VSV (Burkhart et al., 1994b). CD4+ T cells are also required for the establishment and maintenance of CD8+ T cell memory in VSV infected mice (Ramsburg et al., 2007).

Several *in vitro* studies were done showing that VSV stimulated cytotoxic CD4+ T cell responses (Browning et al., 1990a; Browning et al., 1990b; Cao et al., 1993). However, these studies contained flaws that raise serious doubts about their results. First, these studies used immortalized T cell lines and hybridomas made from T cells harvested from VSV-infected mice rather than assaying the T cells directly *ex vivo*. The use of T cell lines and hybridomas indicates that selection processes used when making these T cell lines and hybridomas could have biased the results of these studies. Second, none of these studies included controls for the efficacy of their inhibitors. For example, several intracellular cytokine staining experiments are depicted to demonstrate how the CD4+ T cells resembled a typically CD8+ phenotype, yet no controls were indicated for the efficacy of the Brefeldin A used in the experiments.

1.5.2.4 CD8+ T cell Response to VSV

CD8+ T cells appear to be superfluous for controlling VSV infection in otherwise immunocompetent mice as MHC class I knockout (and thus CD8+ T cell lacking) mice were able to mount an effective immune response to a lethal challenge with VSV (Thomsen et al., 1997). However, in the absence of CD4+ T cells, and thus the absence of neutralizing IgG antibody, CD8+ T cells were able to rescue VSV infected mice from lethality, indicating that CD8+ T cells do play a role in viral clearance, although a redundant one (Andersen et al., 1999; Thomsen et al., 1997). The antigenic determinants that mediate the CD8+ T cell response are derived from the VSV glycoprotein, G, and the nucleoprotein, N (Hale et al., 1978; Lefrancois and Lyles, 1983; Puddington et al., 1986). Emphasizing the significance of the CD4+ T cell response over the CD8+ T cell response to VSV, more CD4+ T helper cell epitopes have been characterized than CD8+ T cell epitopes (Kundig et al., 1993a).

While the primary CD8+ T cell response to VSV appears to be superfluous, memory CD8+ T cell responses may be essential for long-term immunity to VSV. Neutralizing antibody responses to VSV are directed against the glycoprotein, G (Kelley et al., 1972; Lefrancios and Lyles, 1982; Luo et al., 1988). However, these neutralizing antibodies are only reactive to the serotype, or strain, of VSV to which they encounter; no cross-reactivity has been demonstrated (Keil and Wagner, 1989; Luo et al., 1988; Luo et al., 1990). In contrast to the neutralizing antibody response, CD8+ T cells are cross-reactive and are thus able to protect against infection from multiple strains (Burkhart et al., 1994b; Hale et al., 1981; Kundig et al., 1993a; Lefrancois and Lyles, 1983). This implies that CD8+ T cells are more important in the long-term immunity to VSV than they are in the primary immune response.

1.5.3 Vaccinia Virus

Vaccinia virus (VV), a vaccine strain of smallpox derived from cowpox is the prototypal member of the *Orthopoxvirus* genera. Two forms of infectious VV are produced during an infection, intracellular mature virions and extracellular enveloped virions. The majority of the viral progeny produced in a VV infection are intracellular mature virions, which remain inside the cell until cell lysis and lack the lipoprotein envelope (Appleyard et al., 1971; Ichihashi et al., 1971). While extracellular enveloped virions represent only a small proportion of the progeny virus, they are important biologically because they are critical for virus spread in the host (Payne, 1980; Vanderplasschen et al., 1998). Extracellular enveloped virions are released into the extracellular space before cell death (Appleyard et al., 1971). The extracellular enveloped virions also contain an additional lipid membrane, which contains cellular proteins and at least 5 VV-encoded polypeptides that are absent from intracellular mature virions (Payne, 1978; Smith and Vanderplasschen, 1998; Smith et al., 2002b; Vanderplasschen et al., 1998). Both VV virions appear as smooth rounded rectangles often described as "brick-like" (Dubochet et al., 1994). A 30nm membrane-delimited surface layer surrounds viral core of both the intracellular mature virions and the extracellular enveloped virions. An additional lipoprotein envelope surrounds extracellular VV virions (Payne, 1978). VV has a linear double-stranded DNA genome of approximately 195kbp (Baroudy and Moss, 1982). The length of the inverted terminal repeats vary according to strain; the strain of VV used in our experiments, Western Reserve, possesses inverted terminal repeats of 10.5-12kbp (Baroudy et al., 1982). Numerous virus-encoded enzymes, including a multi-subunit DNA-dependent RNA polymerase, a transcription factor, capping and methylating enzymes, and a poly(A) polymerase, are packaged within the virus core (Moss, 1996). VV does not have a known receptor for cell attachment and entry, although the wide host range of VV suggests that the putative entry receptor must be highly conserved. While

the entry receptor is unknown, there is evidence that both the intracellular mature virions and the extracellular enveloped virions fuse with the cell membrane at a neutral pH (Doms et al., 1990). Following entry of VV into the cytoplasm, the virus cores synthesize mRNA and then undergo a second uncoating step (Joklik, 1964). VV, like all poxviruses, is able to replicate entirely within the cytoplasm of the infected cell. Initially, only the early genes are transcribed as they encode proteins involved in stimulation of the growth of neighboring cells, defense against host immune responses, replication of the viral genome, and transcription of the intermediate class of viral genes (Kates and McAuslan, 1967; Munyon et al., 1967). After replication of the viral genome, the progeny viral DNA serve as templates for the successive expression of the intermediate and late genes (Pennington, 1974). Specific viral proteins recognize the distinct sequence elements of the three temporal classes of promoter: early, intermediate and late, providing the basis for a programmed cascade of gene regulation (Baldick et al., 1992; Davison and Moss, 1989a; Davison and Moss, 1989b; Moss). Upon synthesis of the structural proteins under the control of the late promoter, infectious particles are assembled. Particles destined to become extracellular enveloped virions are then wrapped with an additional Golgi derived membrane (Hiller and Weber, 1985; Schmelz et al., 1994). Extracellular enveloped virions are then transported to the periphery of the cell (Cudmore et al., 1995; Stokes, 1976) where they then bud through the plasma membrane and either remain attached to the cell membrane (Blasco and Moss, 1992) or are released into the media (Payne, 1980).

Infection of tissue culture cells with VV results in profound changes in the host cell. Secretion of the VV growth factor (VGF) induces rapid cell growth in the cells surrounding the infected cell (Bablanian, 1970). VGF is capable of binding to the EGF receptor, stimulating autophosphorylation and inducing anchorage independent cell growth (Chang et al., 1988; King et al., 1986; Stroobant et al., 1985; Twardzik et al., 1985). Deletion of VGF has no effect on the infectivity or the viral yield *in vitro* (Buller et al., 1988a), but *in vivo* experiments revealed

diminished replication of the deletion mutant as well as attenuated pathogenicity (Buller et al., 1988b). In addition to changes in the growth patterns of infected tissue culture cells, VV also induces changes in membrane permeability (Carrasco and Esteban, 1982) and inhibits host cellular DNA, mRNA and protein synthesis (Buller and Palumbo, 1991). Inhibition of host DNA replication may occur by hydrolysis of nascent single stranded DNA by a viral endonuclease present in the incoming virion (Dales, 1990; des Gouttes Olgiati et al., 1976). Host protein synthesis decreases gradually after VV infection and is completely shut off around 6 hours post-infection (Buller and Palumbo, 1991). Following expression of VV early genes, translation of cellular mRNA is selectively inhibited by small non-translated polyadenylated mRNAs (Bablanian et al., 1991; Lu and Bablanian, 1996). Additionally, the overall population of cellular mRNAs is reduced progressively, being replaced by VV mRNAs (Becker and Joklik, 1964; Sebring and Salzman, 1967), which contributes to the predominant synthesis of VV polypeptides especially at late times post-infection. The reduction in the population of host mRNAs, could be due to a decrease in RNA polymerase II activity (and thus cellular mRNA synthesis) and to higher degradation rate of all mRNAs that occurs during VV infection (Pedley and Cooper, 1984; Rice and Roberts, 1983). Obviously, the inhibition of host mRNA synthesis and transport greatly affects protein synthesis (Boone and Moss, 1978; Cooper and Moss, 1979). Some experiments suggest that inhibition of host protein synthesis does not require viral gene expression, implicating a protein present in the VV virion (Mbuy et al., 1982; Person-Fernandez and Beaud, 1986) while other experiments suggest that viral transcription is necessary for the inhibition of host protein synthesis (Bablanian et al., 1981; Beaud and Dru, 1980; Mss and Filler, 1970). In yet other experiments, it was shown that inhibition of host protein synthesis is incomplete until after DNA replication (Gershowitz and Moss, 1979; Rosemond-Hornbeak and Moss, 1975) in certain cell types. Thus, inhibition of cellular protein synthesis by VV *in vivo* is likely dependent on the multiplicity of VV, cell type and the time at which the analysis is performed.

VV, like other poxviruses, encodes multiple proteins that interfere with the induction of the host immune response, preventing the activity of complement as well as that of the principle inflammatory cytokines interferons (IFN). TNF and IL-1. VV encodes a protein, VCP, which inhibits the classical and alternative pathways of complement by its ability to bind and inactivate complement (Kotwal et al., 1990; McKenzie et al., 1992). Both type I and type II IFN contribute to the host defense against VV (Friedman et al., 1962; Huang et al., 1993; Karupiah et al., 1993; Schellekens et al., 1981), yet VV is relatively resistant to IFN due to three modes of IFN regulation. In the first mode of IFN regulation, VV encoded inhibitors of the double stranded RNA-dependent protein kinase, PKR, leads to the phosphorylation of the eukaryotic translation initiation factor eIF-2α and thus inhibition of protein synthesis, including that of IFN (Beattie et al., 1991; Carroll et al., 1993; Chang et al., 1992b; Davies et al., 1993; Davies et al., 1992; McCormack et al., 1992). The second mode of IFN regulation is due to aberrant transcription resulting in enhanced degradation of cellular mRNA and rRNA through the 2-5A/RNaseL pathway (Bayliss and Condit, 1993; Paez and Esteban, 1984; Rice and Kerr, 1984; Rice et al., 1984; Whitaker-Dowling and Youngner, 1984). The third mode of IFN regulation involves the secretion of an IFN-γ receptor homolog (encoded by B8R) to bind up and prevent IFN-γ activity (Massung et al., 1993; Shchelkunov et al., 1993). VV also utilizes this third mode to regulate both TNF and IL-1 responses. A soluble viral TNF receptor (encoded by A53R) (Smith et al., 1991) and a soluble viral IL-1β receptor (encoded by B15R) (Alcami and Smith, 1992; Smith and Chan, 1991; Spriggs et al., 1992a), are able to bind TNF and IL-1 respectively and neutralize their activity (Smith, 2000). In addition to the secreted receptor homologs, VV also encodes a Serine Protease Inhibitor, called SPI-2, which inhibits the intracellular conversion of the inactive form of IL-1β to the active secreted form of IL-1β, thus preventing IL-1 function (Palumbo et al., 1989; Pickup et al., 1986; Ray et al., 1992).

1.5.4 Immune Response to Vaccinia Virus

The immune response to VV infection is biphasic, with innate effectors such as interferons (IFN), NK cells and macrophages being crucial in the early phase and with the adaptive, antigen-specific T and B cells developing later to provide clearance of VV and long-term immunity. Each facet of the immune response to VV will be discussed below.

1.5.4.1 Innate Immunity to VV

The innate immune response to VV is dominated by interferons (IFN). IFN are a group of secreted proteins that induce an antiviral state in infected or uninfected cells (Samuel, 1991). There are two groups of IFN: type I IFN are secreted from leukocytes and fibroblasts and include IFN-α and IFN-β. Type I IFN offer resistance to virus infection, stimulate the expression of MHC class I molecules on the surface of the cell and inhibit cell proliferation (Biron, 1998). Additionally, several recent reports indicate that the ability to cross-prime CD8 T cell responses is linked to induction of type I IFN production, suggesting that antigen expressed by VV in our experiments should be able to access the cross-priming pathway (Ahonen et al., 2004; Beignon et al., 2003; Cho et al., 2002; Lapenta et al., 2006). Type II IFN, or IFN-γ, is secreted from macrophages, NK cells and T lymphocytes. Type II IFN is important in the activation of immune and inflammatory responses and for cell-mediated immunity (Farrar and Schreiber, 1993). Both type I and type II IFN are crucial for the restriction of pox virus infections (Huang et al., 1993; Muller et al., 1994; Rodriguez et al., 1991; Schellekens et al., 1981; van den Broek et al., 1995). Mice deficient in IFN receptors are abnormally susceptible to VV infection (van den Broek et al., 1995), showing the importance of these molecules in controlling VV infection. VV also employs three distinct modes of regulating the IFN response, further emphasizing the importance of IFN in

controlling VV infection (Friedman et al., 1962; Huang et al., 1993; Karupiah et al., 1993; Schellekens et al., 1981).

VV infection also stimulates the complement pathway. The complement system of proteins present in the plasma is able to destroy enveloped virions or infected-cells via the classic (Ab-dependent) or alternative (Ab-independent) pathways. The importance of complement in the host defense against VV infection is underscored by the fact that VV encodes a protein, VCP, which binds to and prevents the activity of the complement pathway (Kotwal et al., 1990; McKenzie et al., 1992).

The innate immune response to VV also includes infiltration of the site of infection with neutrophils, macrophages and NK cells. Neutrophils and macrophages are responsible for limiting the initial VV infection by engaging in phagocytosis of infected cells and secreting antiviral and inflammatory cytokines such as TNF and IFN. The importance of macrophages in the immune response to VV infection was demonstrated when mice depleted of macrophages were unable to control VV infections due to impaired virus clearance and antigen presentation (Karupiah et al., 1996). NK cells are also attracted to the site of infection as part of the inflammatory response. The function of NK cells is to kill virus-infected cells which are identified by reduced levels of MHC class I on their surface (Brutkiewicz et al., 1992; Bukowski et al., 1983).

Recently, a role for Toll-Like Receptors (TLR) in the innate immune response to VV was implied by the discovery that a VV protein, A52R, can antagonize TLR signaling (Bowie et al., 2000). TLR are pattern recognition receptors that are required for cellular responses to some viruses and can trigger antiviral signaling pathways leading to IFN responses and maturation of dendritic cells (Bowie and Haga, 2005; Vaidya and Cheng, 2003). Further experiments revealed that A52R blocks NF-κB activation by multiple TLR, including TLR4, by associating with

IRAK2 and/or TRAF6, molecules involved with TLR signaling (Bowie et al., 2000; Harte et al., 2003).

1.5.4.2 VV Humoral Immunity

VV specific memory B cells, responsible for the long-term humoral immunity to VV, have been demonstrated to contribute to human immunity to smallpox, both in their ability to rapidly respond to infection and by their ability to replenish long-lived plasma cells to maintain long-term serum antibody levels (Bernasconi et al., 2002; Crotty et al., 2003). Indeed, VV-specific memory B cells were detected in most human vaccines, even up to 75 years post-immunization (Crotty et al., 2003; Hammarlund et al., 2003). Titers of VV-specific antibodies in immunized humans could also be detected up to 75 years post-immunization in the same range as that observed between 1 and 7 years after vaccination (Crotty et al., 2003; el-Ad et al., 1990; Hammarlund et al., 2003). Most studies use a neutralizing antibody assay that measures whether antibodies in the serum of immunized individuals can prevent infection of tissue culture cells without examining the type of antibody (Mack et al., 1972). However, several studies have demonstrated that several proteins expressed on the surface of intracellular mature virions are the targets of neutralizing antibodies *in vitro*, including A27 (Rodriguez et al., 1985), L1 (Ichihashi and Oie, 1996; Wolffe et al., 1995), H3L (Davies et al., 2005; Lin et al., 2000), and D8 (Hsiao et al., 1999). Mice immunized with recombinant A27 were also protected from lethal intraperitoneal infection with vaccinia virus (Lai et al., 1991), demonstrating that these *in vitro* results may be relevant *in vivo*. Neutralizing antibodies to the B5R protein on extracellular enveloped virions have been demonstrated to be important in controlling VV infection *in vitro* and *in vivo* for both mice (Galmiche et al., 1999; Hooper et al., 2003) and humans (Bell et al., 2004).

1.5.4.3 CD4+ T cell Response to VV

CD4+ T cells play an important role in establishing B cell responses and CD8+ T cell memory responses. Thus, one would expect that the adaptive immune response to VV would require a CD4+ T cell response. VV-specific CD4+ T cells are easily detected following a single immunization with VV (Littaua et al., 1992). In VV immunized humans, VV-specific $CD4^+$ T cells were observed in vaccinees up to 75 years post-immunization; but unlike the memory B cells generated from a single immunization with VV in humans, the number of VV-specific CD4+ T cells declined over the decades, with an estimated half-life of approximately 15 years (Crotty et al., 2003; Hammarlund et al., 2003). In mice, CD4+ T cells were demonstrated to be necessary for antibody mediated clearance of VV (Xu et al., 2004), suggesting that B cell responses to VV require CD4+ T cell mediated help. CD4+ T cells were also required for the establishment of memory CD8+ T cell responses in mice, but were unnecessary for the establishment of the primary CD8+ T cell response (Xu et al., 2004).

1.5.4.4 CD8+ T cell Response to VV

Like CD4+ T cells, VV-specific CD8+ T cells are readily detected following a single round of VV immunization (Littaua et al., 1992) and persist for decades (Demkowicz et al., 1996; Hammarlund et al., 2003). Similar to memory CD4+ T cells, the numbers of memory CD8+ T cells in humans decline over the decades with a half-life of approximately 8-15 years (Demkowicz et al., 1996; Hammarlund et al., 2003). While CD8+ T cell responses have been demonstrated to control VV infection, they may not be required for survival of the host (Hammarlund et al., 2003; Xu et al., 2004). Experiments in β_2-microglobulin knockout mice demonstrated that in the absence if CD8+ T cells, mice were able to survive infection with high

doses of VV in a similar manner to wild-type mice (Spriggs et al., 1992b). Additional data from Xu *et al.* demonstrated that VV-specific CD8+ T cell responses were only necessary in the absence of either a humoral response or CD4+ T cells (Xu et al., 2004), as CD8+ T cell depleted or knockout mice were able to mediate a primary immune response. Even the requirement for VV-specific memory CD8+ T cell responses is doubtful as studies in both humans (Demkowicz et al., 1996; Littaua et al., 1992) and chimpanzees(Zarling et al., 1987) have demonstrated the perforin-dependent lysis of VV-infected cells by CD4+ memory T cells. Together, these results suggest that immunity to VV may be more dependent on CD4+ T cells than on CD8+ T cells.

1.6 Focus of this Thesis

T cells play an essential role in the protective immunity to viruses and tumors, yet little is known about how T cell immunity is activated *in vivo*. In order to better stimulate T cell immunity by vaccines, a better understanding of the *in vivo* antigen processing and presentation pathways is needed. Therefore, the focus of the experiments presented in this thesis is the elucidation of the pathways of T cell activation *in vivo*. Several hypotheses were tested in this dissertation:

1. The 8-10 residue peptides presented on MHC class I can be derived from endogenously synthesized proteins, a process known as direct priming, or derived from exogenous proteins synthesized by other "donor" cells and taken up by the antigen presenting cell, a process known as cross-priming. Designing vaccines to take advantage of the cross-priming pathway of T cell activation requires knowledge of the physiologically relevant form of antigen transferred from the antigen donor cell to the antigen presenting cell *in*

vivo. In Chapter 3, the form of antigen transferred in cross-priming *in vivo* was determined by probing whether whole protein, minimal antigenic peptides or degradation intermediates could be transferred from a presentation-incompetent donor cell to the antigen presenting cell *in vivo*.

2. To enhance the donation of antigen to the cross-priming pathway *in vivo,* it is necessary to determine which cellular processes are required in the antigen donor cell for donation of antigen. The requirement for cellular processes such as proteasomal degradation, protein synthesis and secretory function in the antigen donor cell were all examined in Chapter 3. In addition, Chapter 3 also examined the requirement for new synthesis of antigen *in vivo* for both the cross- and direct priming pathways.
3. Recombinant viral vectors expressing transgenes from other diseases have been proposed as a means of stimulating CD8+ T cell immunity to the transgene *in vivo*. Studies have demonstrated that recombinant viral vectors can effectively express and stimulate immunity to a transgene (Cooney et al., 1991; Kahn et al., 2001; Konishi et al., 1998; Ockenhouse et al., 1998; Ramsburg et al., 2004; Reuter et al., 2002; Roberts et al., 1998; Roberts et al., 2004; Rose et al., 2001; Schlereth et al., 2000). However, systematic examination of the ability of virally expressed antigen to access the antigen processing and presentation pathways *in vivo* is required to enhance vaccine design using recombinant viral vectors. In Chapter 4, we examined the ability of a model antigen, ovalbumin, to access the MHC class I antigen processing and presentation pathways *in vivo* when expressed by either recombinant Vaccinia Virus or recombinant Vesicular Stomatitis Virus.
4. While running controls for our experiments in Chapter 4, we observed transporter-associated with antigen processing (TAP)-dependent MHC class II presentation of OVA. TAP, which transports poly-peptides into the ER for presentation on MHC class I, is not

typically thought to be involved in MHC class II processing as MHC class II molecules are typically prevented from binding peptides in the ER by the invariant chain (Roche and Cresswell, 1990; Teyton et al., 1990). However, other groups have reported a requirement for TAP in the transport of cytosolic-processed MHC class II peptides (Carmichael et al., 1996; Malnati et al., 1992; Tewari et al., 2005). Therefore, in Chapter 5, we examined the ability of ovalbumin to access the MHC class II antigen processing and presentation pathways *in vivo* when expressed by either recombinant Vaccinia Virus or recombinant Vesicular Stomatitis Virus.

Chapter 2

Materials and Methods

2.1 Mice

OT-I TCR RAG1 (Hogquist et al., 1994; Mombaerts et al., 1992), OT-II TCR (Barnden et al., 1998), and Bg1 TCR (Donohue et al., 2006) transgenic mice were obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Exchange Program (Line 4175; NIAID, Bethesda, MD). B6.SJL-Ptprca/BoAiTac mice were purchased from Taconic Farms (Germantown, NY) and bred with OT-I TCR RAG1 or OT-II TCR transgenic mice to produce the OT-I.SJL or OT-II.SJL offspring respectively that were used in the indicated adoptive transfer experiments. Female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). H-2K^{bm1} (B6.C-H2bm1/By) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specified pathogen-free conditions at Penn State Hershey Medical Center under the Institutional Animal Care and Use Committee guidelines.

2.2 Viruses

2.2.1 Recombinant Vaccinia Virus and Influenza A Virus

The rVVs and Influenza A Virus (IAV) used were produced, propagated, and used to infect cells as described previously (Basta et al., 2002; Deng et al., 1997; Overwijk et al., 1997; Restifo et al., 1995) and were a kind gift from Drs. Jon Yewdell and Jack Bennink (Laboratory of

Viral Diseases, NIAID, Bethesda, MD). The antigenicity of rVVs encoding $OVA_{257\ 264}$-containing gene products have been characterized in a previous publication (Bacik et al., 1994). Notably, rVVs that express minigenes are much more efficient at producing K^b-$OVA_{257-264}$ complexes than rVVs that express NP-S-GFP. The rapidly degraded form of NP-S-GFP produces complexes 3 times as efficiently as the stable form. This rapidly degraded form is a chimeric protein consisting of ubiquitin genetically fused to the amino terminus of NP-S-GFP whose initiating Met is altered to Arg. Ubiquitin is removed co-translationally leaving a classic "N-end rule" substrate (Varshavsky, 1992).

2.2.1.1 Vaccinia Virus Purification

Where rVV were purified, the infected cells were pelletted at 1100 x g for 10 minutes and the supernatant was aspirated. The pellet was then resuspended in ice-cold 10mM Tris pH 9.0, and the cells were permitted to swell on ice for 15 minutes. The cell suspension was transferred to a Dounce homogenizer and homogenized to rupture the cytoplasmic membranes. The homogenate was then centrifuged at 105 x g for 5 minutes at 4°C to remove cell nuclei. Supernatant was collected and placed aside while the pellet was resuspended in ice-cold 10mM Tris pH 9.0 as above and re-homogenized. Supernatant from the second homogenization was combined with supernatant from the initial homogenization layered on top of a 45% sucrose cushion. The preparation was centrifuged at 25,000 x g for 90 minutes at 4°C. The supernatant was collected and titrated before being aliquoded and stored at -80°C until use.

2.2.1.2 Preventing Host Infection from Vaccinia Virus-Infected Cells

To prevent the infection of hosts with rVV released from infected cells, it was necessary to UV irradiate infected cells prior to their introduction into mice. We used two protocols for this purpose. For protocol one, cells were incubated with 10µg/mL psoralen [4,5',8-Trimethylpsoralen (Sigma, St Louis, MO)] for 10 minutes, then exposed to long-wave (366-nm) UV irradiation for 20 minutes. Inactivation reduced viral titers to less than 1 PFU per $2x10^7$ cells, and prevented antigen presentation from the lysate of infected cells when exposed to L-K^b cells, as measured by activation of an $OVA_{257-264}$-specific hybridoma. For protocol two, rVV-infected cells were resuspended at a concentration of $1.5x10^7$cells/mL in HBSS/0.1%BSA and irradiated on ice for 60 minutes with 254-nm light at a flux of 4.28 mW/cm^2. In cases where infected cells were fixed, the 30 minute incubation in 2% paraformaldehyde at room temperature was used instead of psoralen/UV treatment to prevent transfer of rVV to the host (Hulskotte et al., 1997).

2.2.1.3 Expression of H2-K^b in L4.2 cells using Vaccinia Virus

Where rVV was used to express H2-K^b in L4.2 cells, cells were infected with a multiplicity of infection (MOI) = 10 of rVV-K^b, and incubated for 30 min with gentle agitation every 5 min. After 30 min of infection, medium containing 40µg/mL cytosine 1-β-D-arabinofuranoside (AraC) (Sigma, St Louis, MO) was added to infected cells to prevent transcription of β-gal, which is encoded by a late gene in rVV. AraC was present throughout the duration of the experiment.

2.2.2 Recombinant Vesicular Stomatitis Virus

rVSV were produced, propagated, and used to infect cells as described previously (Kim et al., 1998) and were a kind gift from Dr. Leo Lefrancois (University of Connecticut Health Center, Department of Immunology, Farmington, CT). It should be noted that when propagating rVSV, an MOI of 0.025 was used to prevent the accumulation of DI particles in our viral stocks.

2.2.2.1 Vesicular Stomatitis Virus Purification

Where rVSV were purified, the infected cells were pelletted at 83,000 x g for 90 minutes at 4°C and the supernatant was aspirated. The pellet was then resuspended in ice-cold PBS overnight. The cell suspension was then layered on top of a 60%/10% sucrose gradient and centrifuged at 83,000 x g for 90 minutes at 4°C. Virus was harvested from the interface and pelleted at 25,000 x g for 60 minutes at 4°C. Pellets were resuspended in HBSS/0.1%BSA overnight at 4°C. Suspension was then titrated before being aliquoded and stored at -80°C until use.

2.2.2.2 Preventing Host Infection from Vesicular Stomatitis Virus -Infected Cells

Where rVSV were used in cross-priming experiments, infected cells were treated with psoralen/UV irradiation as above. In addition, cells were incubated for 30 minutes with neutralizing antibody (CRL-2700, ATCC) prior to introduction into hosts.

2.2.3 Recombinant Retrovirus Expressing H2-K^b

A Phoenix Ampho packaging cell line producing a retrovirus expressing the murine MHC class I molecule H2-K^b was a kind gift from Dr Peter Cresswell (Department of Immunobiology, Yale University, New Haven, CT).(Ackerman and Cresswell, 2003)

2.3 Cells

2.3.1 Cell lines and cultures

All media were purchased from Invitrogen (Carlsbad, CA). β_2-microglobulin negative cells (laboratory designation: STBKM-1) (Norbury et al., 2004), 293A (Graham et al., 1977) (human cells), P815 (MHC mismatched [H-2^d] cells) (Ralph et al., 1976), WT3 (Pretell et al., 1979) (wild type fibroblasts) and 1E12 (Norbury et al., 2001)(TAP-/- fibroblasts) cells were maintained in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 2mM glutamine. B3Z cells(Shastri and Gonzalez, 1993) and OT2Z cells (a kind gift from Dr. Nilabh Shastri, Division of Immunology, Department of Molecular and Cell Biology, University of California, Berkeley, CA) were cultured in RPMI containing 5% FBS, 1% penicillin-streptomycin, 2mM glutamine and 1% non-essential amino acids. BO-80.10 cells, a kind gift from Dr. Philippa Marrack (Integrated Department of Immunology and Howard Hughes Medical Institute, National Jewish Medical and Research Center, Denver, CO), were cultured in Iscove's containing 5% FBS, 1% penicillin-streptomycin, 2mM glutamine, 1% non-essential amino acids, and 0.0062% β-mercaptoethanol.

2.3.2 Plasmids and transfections for human (293) cells

All plasmids used were based on pcDNA3.1D/V5-His-TOPO (Invitrogen). A plasmid expressing OVAMet$_{257-264}$ (pminiSIIN) was made by annealing two oligodeoxynucleotides, CACCATGAGTATAATCAACTTTGAAAAACTGTGA and

TCACAGTTTTTCAAAGTTGATTATACTCAT, and cloning the product using a pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). GFP-OVA$_{257-264}$ is the plasmid GFPS in (Princiotta et al., 2001). The control plasmid used for this experiment was pcDNA3.1D/V5-His/*LacZ* that is provided with the Directional TOPO Kit (Invitrogen). 293A and 293KbC2 were grown to 90% confluence in 180 cm2 flasks and each flask transfected with 20μg of DNA and 40μL of Lipofectamine 2000 reagent in 12mL of Opti-MEM according to the manufacturer's instructions (Invitrogen). After 2 hours, a further 12mL of DMEM containing 10% FBS was added and transfections left for 22 hours. When more than one flask was transfected with one plasmid, aliquots of a single mix of DNA and Lipofectamine 2000 were used. Transfected cells were harvested with trypsin (as per usual maintenance), spun and resuspended in Hanks' balanced salt solution with 0.5% bovine serum albumin (HBSS/BSA) for immunization of mice or staining with mAbs and flow cytometric analysis.

2.3.3 MHC mismatched (P815) cell infection, characterization, and immunization

P815 cells were treated with 20μM lactacystin for 1 hour prior to infection and then infected at a multiplicity of infection of 2. After 1 hour, cells were washed and resuspended in DMEM containing 10% FBS and incubated for an additional 12-16 hours in the presence of 20μM lactacystin.

2.3.4 Characterization of lactacystin effects on β_2-microglobulin negative cells

Cells vary greatly in their sensitivity to lactacystin. We confirmed that lactacystin inhibits proteasomes in β_2-microglobulin negative cells by infecting cells with a rVV expressing R-NP-OVA$_{257-264}$-GFP (NP-OVA$_{257-264}$-GFP genetically fused to ubiquitin at its NH2-terminus, substituting Arg for the initiating Met of NP; ubiquitin is co-translationally cleaved from this protein, leaving a classic "N-end rule" substrate degraded by proteasomes with a t1/2 of 10min (Varshavsky, 1992). Cells were treated with lactacystin starting 1 hour prior to infection and ending 3 hours post infection. Cells were washed, subjected to sufficient psoralen-long wave UV treatment (Tsung et al., 1996) to prevent transfer of adsorbed virus to host cells following immunization with rVV-infected cells, and then incubated for 12 hours. The amount of fluorescent R-NP-OVA$_{257-264}$-GFP remains constant for at least 12 hours after the removal of lactacystin. Since psoralen-UV irradiation completely inhibits neosynthesis of viral proteins (demonstrated by its affect on cells expressing NP-OVA$_{257-264}$-GFP), the steady-state level of R-NP-OVA$_{257-264}$-GFP in lactacystin withdrawn cells demonstrates the irreversible nature of the proteasome blockade in these cells.

2.3.5 β_2-microglobulin negative cell infection, characterization, and immunization

β_2-microglobulin negative cells were treated with 20μM lactacystin for 1 hour prior to incubation with rVVs at a multiplicity of infection of 10PFU for 1 hour. Cells were then washed and rotated in Iscove's medium containing 10% FBS for 4 hours in the presence or absence of 20μM lactacystin. Mice were immunized via i.p. injection with approximately $3x10^7$ cells.

2.3.6 Creation of L4.2 Cells

The 293 Tetracycline-Regulated Expression (T-REx) cell line stably expressing the tetracycline (tet) repressor was purchased from Invitrogen and cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% FBS, 1% penicillin-streptomycin, 2mM glutamine and 5μg/mL blasticidin. L4.2 cells (H2-K^b negative) were created as follows. pcDNA4/TO/lacZ, a plasmid containing the bacterial gene encoding β-galactosidase (*LacZ*) gene fused to the tetracycline operator and the selectable zeocin resistance gene, was purchased from Invitrogen. 293 T-REx cells (60% confluent) were transfected with 1μg of purified plasmid DNA using lipofectamine as per the manufacturer's instructions (Invitrogen). Stably transfected cells were selected by zeocin (300μg/mL) resistance over the course of 3 weeks. Clonal populations were then isolated by limiting dilution and screened for LacZ activity after induction with 1μg/mL tetracycline. Clonal populations were maintained in DMEM containing 10% fetal calf serum, 1% penicillin-streptomycin, 2mM glutamine, 5μg/mL blasticidin and 300μg/mL zeocin.

2.3.7 Creation of L4.2mK^b cells

L4.2mK^b cells were created from L4.2 cells. L4.2 cells were transduced with a retrovirus expressing the murine MHC class I molecule H2-K^b. Briefly, $5x10^5$ L4.2 cells were plated in a 100mm plate and then infected with the retrovirus in the presence of 4μg/mL polybrene (Fisher, Fairlawn, NJ) for 3 hr. Additional medium was then added and cells were cultured for 48 hr. Retrovirally transduced cells were selected with 0.5mg/mL geneticin (Invitrogen) for 3 weeks. L4.2mK^b cells were then stained with the anti-K^b antibody HB176 and sorted using a MoFlo (Cytomation, Fort Collins, CO) to further select cells expressing K^b. K^b-expressing cells were

maintained in DMEM containing 10% fetal calf serum, 1% penicillin-streptomycin, 2mM glutamine, 5μg/mL blasticidin, 300μg/mL zeocin and 0.5mg/mL geneticin.

2.3.8 Single cell LacZ assay

L4.2 cells were treated with 1μg/mL tetracycline in culture media for 4 hr and β-gal expression was assayed using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D galactopyranoside (X-gal). For staining, cells were harvested using versene, washed once in phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde/0.2% gluteraldehyde for 10 min at room temperature. Cells were washed again in PBS and then overlaid with a solution containing 1mg/mL X-gal, 5mM potassium ferrocyanide, 5mM potassium ferricyanide and 2mM $MgCl_2$ in PBS and incubated overnight at 37°C. Cells were counted using a hemocytometer to determine the number displaying LacZ activity. A minimum of 1000 cells were counted for each of the triplicate samples. (Sanderson and Shastri, 1994)

2.3.9 Bulk LacZ assay

L4.2 cells were harvested and treated with 1μg/mL tetracycline in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum, 1% penicillin-streptomycin, 2mM glutamine and 5μg/mL blasticidin at 37°C for the times indicated. In instances where tetracycline was removed, L4.2 cells were harvested and treated as above for 4 hr. L4.2 cells were then washed twice in IMDM. For time points under 24 hr, cells were resuspended in IMDM, transferred to a fresh tube and placed back on the rotator until the end-point. For times over 24 hr, cells were resuspended in culture media, plated into dishes to prevent the cell death observed when rotated for longer than 24 hr, and then harvested 1 hr prior to the end-point.

Before being returned to dishes, cells were enumerated to allow determination of cell division over the time–course of the experiment. Tetracycline-treated cells rarely divided; after 8 days in culture, less than half of the cells had divided once. At the end-point, cells were harvested, washed twice in PBS, and plated at $1x10^5$ cells/well in a flat-bottomed 96-well plate (Corning, Inc., Corning, NY). Ten-fold dilutions were then performed. Cells were lysed by addition of 200µL of buffer containing 100mM β-mercaptoethanol, 9mM $MgCl_2$, 0.125% IGEPAL (Sigma) and 0.15mM chlorophenol red β-galactoside (CPRG; Calbiochem, San Diego, CA) in PBS. After 10–20 min of incubation at 37°C, 50µl of stop buffer [300mM glycine and 15mM ethylenediaminetetraacetic acid (EDTA) in water] was added to each well, and absorption was read using a Dynex MRX 96-well plate-reader (Dynex, Chantilly, VA) at 595nm with a reference filter of 630nm.

2.3.10 Requirement for protein synthesis in cross-priming *in vivo*

β_2-microglobulin knockout (STBKM-1) cells, which were unable to present the H2-K^b-restricted $OVA_{257-264}$ or β-gal_{96-103} peptides derived from endogenously synthesized proteins, were harvested and treated with 25µg/mL emetine (Sigma) or cycloheximide (Calbiochem). Cells were kept in suspension by gentle continuous rotation for the duration of treatment, a maximum of 5 hr. At the end-point, a suspension of 2–$4x10^6$ cells in PBS containing 10mM $MgCl_2$ and 1mg/mL OVA (Worthington Chemicals, Lakewood, NJ) was electroporated at 0.25 kV in disposable cuvettes using a Bio-Rad gene pulser (Bio-Rad, Hercules, CA). Cells were incubated on ice for 10 min immediately before and after electroporation and then washed three times before being subjected to 20,000 rads of gamma-irradiation. After irradiation, cells were washed once and counted prior to injection. Each batch of OVA used was screened for the presence of contaminating antigenic peptide by incubating with fixed APC and assaying for presentation to an

OVA-specific $CD8^+$ T-cell hybridoma (Reis e Sousa and Germain, 1995). Only batches of OVA with undetectable levels of contaminating peptide were used in our studies. The concentration of OVA was chosen after titration to determine the lowest concentration that generated an antigen-specific $CD8^+$ T-cell response following immunization *in vivo*.

2.3.11 Isolation and *in vitro* culture of Bg1.SJL TCR transgenic T cells

Splenocytes (1×10^7) from Bg1.SJL mice were plated with 5×10^5 of a gamma-irradiated cell line, E22, which stably expresses β-gal. On day 3 after culture set-up, cell debris was removed by centrifugation over LSM and then cells were cultured for an additional 3 days before use. Cultures were either used on day 6 or restimulated with gamma-irradiated E22 cells on day 7 if cultured for longer.

2.3.12 *In vitro* direct presentation

WT3 cells were treated with either 2.5µg/mL emetine or 25µg/mL cycloheximide. Cells were kept in suspension by gentle rotation for the duration of treatment. WT3 cells were then electroporated as described above, with the following exceptions. WT3 cells were electroporated at 0.45 kV in the presence of 10mg/mL OVA (Worthington Chemicals). Following electroporation, cells were washed twice and plated at $1x10^5$ to $5x10^5$ cells/well depending on the conditions examined. WT3 cells treated with cycloheximide were then incubated in the presence of cycloheximide for an additional 5 hr before being washed twice and then fixed or not as indicated. WT3 cells were fixed for 10 min at room temperature in 1% paraformaldehyde (Sigma). Fixed cells were washed twice in PBS, treated with 0.2M glycine (Sigma) and then washed twice more before the addition of B3Z cells to test for antigen presentation *in vitro*.

2.3.13 B3Z Assay

$1x10^5$ stimulator cells were co-cultured overnight with $1x10^5$ B3Z cells, an $OVA_{257-264}$-specific T cell hybridoma which produce β-galactosidase upon activation. The chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D galactopyranoside (X-gal) was used to stain cells as follows. Cells were centrifuged at 235 x g and fixed with 2% paraformaldehyde/0.2% gluteraldehyde for 10 min at room temperature. Cells were washed twice in PBS and then overlaid with a solution containing 1mg/mL X-gal, 5mM potassium ferrocyanide, 5mM potassium ferricyanide and 2mM $MgCl_2$ in PBS and incubated overnight at 37°C. Cells were counted using a hemocytometer to determine the number displaying LacZ activity. A minimum of 1000 cells were counted for each of the triplicate samples. (Sanderson and Shastri, 1994)

2.3.14 Characterization of Brefeldin A treatment effects on viral infection of and protein expression in β_2-microglobulin negative cells.

Reports have indicated that the secretory rearrangements caused by Brefeldin A (BFA) (Sigma) can inhibit viral replication (Cuconati et al., 1998; Doedens et al., 1994; Gazina et al., 2002; Jensen and Norrild, 2002) and protein synthesis (Fishman and Curran, 1992; Mellor et al., 1994). The effects of BFA treatment on rVV infection and protein expression were tested using an rVV expressing a chimeric fluorescent protein (NP-S-GFP). β_2-microglobulin negative cells were infected for 1 hour and then either untreated or treated with 5μg/mL BFA for an additional 4 hours. Protein expression was monitored by GFP fluorescence and was determined by flow cytometry. Infection was determined by the percent of fluorescent cells while protein expression was determined by the mean fluorescence of the cells, which is used as a measure of how much of the chimeric protein was made.

2.3.15 Requirement for vesicular transport in cross-priming *in vivo*

β_2-microglobulin negative cells were treated infected at a multiplicity of infection of 10 PFU for 1 hour. Cells were then washed and rotated in Iscove's medium containing 10% FBS for 4 hours in the presence or absence of 5μg/mL BFA. Cells not treated with BFA were then fixed in 2% paraformaldehyde for 30 minutes at room temperature before being placed on ice. Cells treated with BFA for rotated for an additional hour to compensate for the effect of BFA on protein synthesis before being fixed as above. Mice were immunized via i.p. injection with approximately $3x10^7$ cells.

2.3.16 Isolation and culture of Bone Marrow Dendritic Cells (BMDC)

Femurs were harvested from C57BL/6 or TAP-/- mice. Bone marrow was then flushed out of each femur using 10mL of DC media (Iscove's containing 10% FBS, 1% penicillin-streptomycin, 2mM glutamine and 10%GM-CSF supernatant) pushed through a 23 gauge needle. Bone marrow from 2 femurs was placed in a 50mL conical and centrifuged at 235 x g. Pellet was then resuspended in 6mL of DC media and 1mL of marrow was added to each well of a 6-well plate (Corning Inc., Corning, NY). Each well then received an additional 3mL of DC media. Plates were swirled every other day, with 2mL of media being replaced with fresh media. BMDC were harvested for use on day 6.

2.3.17 BMDC Purification

Where BMDC were purified for CD11c, BMDC were cultured and harvested as above. A purified CD11c population was generated by incubating with anti-CD11c (N418) beads

(Miltenyi Biotech, Auburn, CA) for 15 minutes on ice, then isolating positive cells using the AutoMACS system.

2.3.18 Reconstitution of cross-priming using OVA electroporated cells.

β_2-microglobulin negative cells were mock infected or infected with a multiplicity of infection of 10PFU with either rVV or rVSV for four hours. Cells were then washed twice and electroporated with OVA as above (Requirement for protein synthesis). However, rather than be treated with gamma-irradiation to prevent cell division, the cells were treated to prevent transfer of infectious virus to the host.

2.3.19 Isolation and *in vitro* culture of OT-II.SJL TCR transgenic T cells

Splenocytes ($1x10^7$) from OT-II.SJL mice were plated with $2x10^7$ gamma-irradiated (10,000rads) C57BL/6 splenocytes pulsed with 1mg/mL OVA (Worthington Chemicals) in CD4 T cell Media (RPMI containing 10% FBS, 1% penicillin-streptomycin, 2 mm glutamine, 1% non-essential amino acids and 30u/mL IL-2). On day 3 after culture set-up, cell debris was removed by centrifugation over LSM and then cells were cultured for an additional 3 days before use. Cultures were either used on day 6 or re-stimulated with gamma-irradiated, 1mg/mL OVA pulsed C57BL/6 splenocytes on day 7 if cultured for longer.

2.3.20 CD4 Purification of cultured OT-II.SJL cells

Where OT-II.SJL cells were selected for CD4, OT-II.SJL cells were initially either cultured as above for 6 days or purified according to the protocol for adoptive transfers (see

below). A purified CD4 population was generated by incubating with anti-CD4 (L3T4) beads (Miltenyi Biotech, Auburn, CA) for 15 minutes on ice, then isolating positive cells using the AutoMACS system.

2.4 Purification and adoptive transfer of TCR transgenic T cells

Splenocyte populations were obtained from OT-I TCR RAG1, OT-I.SJL TCR, Bg1.SJL TCR or OT-II.SJL TCR transgenic as follows. Lymph nodes (popliteal, inguinal, brachial, axillary and superficial cervical) and spleen were removed, homogenized and centrifuged over lymphocyte separation medium (LSM; Cambrex, Walkersville, MD) to yield mononuclear cells, which were injected intravenously. For analysis of cell division, TCR transgenic cells were washed in PBS, then incubated with 5µM CFDA-SE (Molecular Probes, Eugene, OR) for 10 min at 37°C, and washed twice in IMDM prior to injection. Between 5×10^6 and 1×10^7 cells were injected into recipients.

2.5 Flow cytometric analysis

All antibodies were purchased from BD Biosciences (San Jose, CA) unless otherwise indicated, and flow cytometry analysis was performed on a FACSCanto, FACS Calibur or FACScan (BD Biosciences). Data was analyzed using FlowJo software (Treestar, San Carlos, CA). Cells were incubated in 2.4G2 (anti-CD16, -CD32) supernatant containing 10% mouse serum (Sigma) for 20 min on ice to block Fc receptor-mediated uptake of antibody.

2.5.1 Analysis of $OVA_{257-264}$ peptide binding

Splenocytes from $H2^b$ or $H2^{bm1}$ mice were incubated at 26 °C with FITC-conjugated $OVA_{257-264}$ peptide for 40 minutes and washed extensively prior to analysis.

2.5.2 Analysis of activation marker expression

Spleens were harvested from two mice per group and homogenized and the cells were pooled. Mononuclear cells were isolated by centrifuging over LSM and harvesting the cells at the LSM/medium interface, then stained with anti-Vα2-PE (clone B20.1.1), anti-CD8-PECy5 (clone 53-6.7), and anti-CD69-FITC (Clone H1.2F3).

2.5.3 Analysis of cell division *in vivo*

Spleens were harvested and mononuclear cells isolated as above. Mononuclear cells were then stained with either anti-Vα2-PE (clone B20.1.1) (for identifying adoptively transferred OT-I TCR RAG1 cells) or anti-CD45.1-PE (clone A20) (for identifying adoptively transferred OT-I.SJL, Bg1.SJL or OT-II.SJL cells) and anti-CD8-PECy5 (clone 53-6.7). Cells were washed three times prior to data capture. Only Vα2/CD45.1, CD8 double positive cells were analyzed for CFDA-SE staining.

2.5.4 Analysis of CD8+ T cell cytokine production

Mononuclear cells harvested isolated from spleens as above were resuspended in RPMI containing 10% FBS at $2x10^7$ cells/mL, and 100µL were added per well to round-bottom 96-well

plates. Cells were incubated for 2 hours at 37°C following addition of synthetic peptide (0.5μM) and then for a further 3 hours following the addition of Brefeldin A (10μg/ml). Cells were stained with anti-CD8α-PE-Cy5 (clone 53-6.7) for 60 min on ice, washed and then fixed in 1% paraformaldehyde for 10 min at 25°C. Lymphocytes were then stained with anti-mouse interferon (IFN)-γ-fluorescein isothiocyanate (FITC) (XMG1.2) in Fc block containing 0.5% saponin and 10% mouse serum for 60 min on ice. Data are expressed as the percentage of $CD8^+$ T cells that produce IFN-γ.

2.5.5 Analysis of cell division *in vitro*

Cultured OT-II.SJL cells or CD4 purified OT-II.SJL cells isolated according to the adoptive transfer protocol were labeled with 5μM CFDA-SE (Molecular Probes, Eugene, OR) for 10 min at 37°C, and washed twice in IMDM. $1x10^5$ CFDA-SE-labeled CD4+ OT-II.SJL cells were then plated out with an equal number of stimulator cells in a 96-well dish with varying conditions being plated in triplicate. Cells were co-incubated for 96 hours. Cells were then spun down and stained with anti-CD45.1-PE (clone A20) (for identifying OT-II.SJL cells) and anti-CD4-PECy5 (L3T4). Cells were washed three times prior to data capture. Only CD45.1, CD4 double positive cells were analyzed for CFDA-SE staining.

2.5.6 Analysis of CD4+ T cell cytokine production

For analysis of CD4+ T cell cytokine production, mononuclear cells harvested isolated from spleens as above were resuspended in RPMI containing 10% FBS at $2x10^6$ cells/mL, and 100μL were added per well to round-bottom 96-well plates. Cells were incubated for 8 hours at 37°C following addition of synthetic peptide (10μM) and then for a further 16 hours following

the addition of Brefeldin A (10μg/mL). Cells were stained for 20 min on ice with anti-CD4-Alexa647 (L3T4) (eBiosciences, San Diego, CA) for 60 min on ice, washed and then fixed in 1% paraformaldehyde for 10 min at 25°C. Lymphocytes were then stained with anti-mouse IFN-γ-fluorescein isothiocyanate (FITC) (XMG1.2) and anti-mouse IL-2- PE (JES6-5H4) in Fc block containing 0.5% saponin and 10% mouse serum for 60 min on ice. Data are expressed as the percentage of $CD8^+$ T cells that produce IFN-γ or IL-2.

2.6 Western Blots

2.6.1 Extent of OVA Expression

$1x10^5$ fibroblasts were infected with an MOI=10 with rVV or rVV-OVA for 4 hours or with rVSV or rVSV-OVA for 16 hours. For cells that were treated with Brefeldin A, 5μg/mL Brefeldin A was added 1 hour post-infection for rVV or 4 hours post-infection for rVSV. Cells were spun down after infection and pellets were resuspended in 20μL lysis buffer (9mM $MgCl_2$, 0.125% IGEPAL, and 0.35% β-mercaptoethanol in PBS) and incubated for 20 minutes at room temperature. rVV or rVV-OVA infected cells were then diluted 1:1000 in lysis buffer. For samples where supernatant was concentrated, supernatant was harvested and loaded onto a Centriprep Ultracel YM-30 Centrifugal filter device (Millipore, Billerica, MA) and centrifuged according to manufacturer's protocol. 20μL of loading buffer (4% SDS, 50mM Tris/HCL pH 6.8, 12% glycerol, 0.01% Bromophenol Blue and 2% β-mercaptoethanol) was then added to concentrated supernatant or cell pellet solution and samples were incubated for an additional 15 minutes at 95°C. Samples were then loaded onto a 4-12% NuPage Bis Tris Precast Gel (Invitrogen) and run in NuPage MOPS SDS running buffer (Invitrogen). Proteins were then

transferred to Hybond P nitrocellulose membrane at 4°C in NuPage MOPS SDS running buffer containing 20% methanol. Membranes were then blocked for 1 hour in 5% BSA (Fraction V, Sigma) in PBS-0.1%Tween. Primary antibody (anti-OVA-IgG, Rockland, Gilbertsville, PA) incubation was carried out overnight at 4°C. Membranes were then washed 4 times in 2.5%BSA in PBS-0.1%Tween. Membrane was then blocked in 2.5% milk in PBS-0.1% Tween for 15 minutes before addition of secondary antibody (anti-mouse/rabbit from BM Chemiluminescence Western Blotting Kit, Roche, Penzberg, Germany) and further incubation at room temperature for 30 minutes. Blots were then developed using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL).

2.6.2 Amount of OVA in Viral Preps

20uL of virus from unpurified or purified viral preps with incubated with 20μL of loading buffer (4% SDS, 50mM Tris/HCL pH 6.8, 12% glycerol, 0.01% Bromphenol Blue and 2% β-mercaptoethanol) for 15 minutes at 95°C before being loaded onto a 4-12% NuPage Bis Tris Precast Gel and blotted as above.

2.7 ELISA

Stimulator cells (either uninfected, infected or peptide pulsed) were plated out at $2x10^5$cells/well in a 96-well plate and incubated with either $1x10^5$ CD4 purified OT-II.SJL cells or $OVA_{323-339}$-specific hybridoma cells, BO-80.10. Cells were co-incubated for 24-96 hours. 24 hours was sufficient to detect IL-2 production in response to $OVA_{323-339}$-pulsed cells, however longer time points were used to verify the lack of response seen to rVV-OVA or rVSV-OVA infected cells. After co-incubation, plates were spun down and supernatant was harvested and

used in an IL-2 ELISA. The IL-2 ELISA was performed using a Mouse IL-2 ELISA Ready-SET-Go kit (eBiosciences) according to manufacturer's protocol. ELISA plates were read using a Dynex MRX 96-well plate-reader (Dynex, Chantilly, VA) at 450nm with a reference filter of 570nm.

Chapter 3

Cross-Priming Utilizes Antigen Not Available to the Direct Presentation Pathway

3.1 Introduction

CD8+ T cells play a critical role in immunity by eliminating virally infected cells, tumors and transplanted tissues. CD8+ T cells recognize 8-10 amino acid residue peptides presented on MHC class I molecules. The 8-10 residue peptides presented on MHC class I can be derived from endogenously synthesized proteins, a process known as direct priming, or derived from exogenous proteins synthesized by other "donor" cells and taken up by the antigen presenting cell, a process known as cross-priming. To efficiently target each of these antigen-processing pathways during rational vaccine design, it is necessary to characterize the requirements of these antigen-processing pathways *in vivo*.

Direct priming studies are almost exclusively carried out *in vitro* in order to exclude the contributions of the cross-priming pathway. While the direct priming pathway can utilize stable cellular proteins, defective ribosomal products (DRiPs) are hypothesized to be the main source of antigenic peptide in the class I pathway (Yewdell et al., 1996). According to the DRiP hypothesis, ribosomes are error prone and defective translation products are an important source of peptides for MHC class I molecules (Yewdell et al., 1996). DRiPs are thought to be generated from newly synthesized polypeptides that are unable to reach their native state due to mistranslations, truncations, improper folding, or improper post-translational modifications.

DRiPs are rapidly degraded (Reits et al., 2000; Schubert et al., 2000) and constitute at least 30% of all proteins synthesized (Schubert et al., 2000), although they can only be visualized under conditions in which their degradation is inhibited. With DRiPs making up at least 30% of all proteins synthesized, they permit the presentation of peptides from newly synthesized bacterial and viral products, leading to a more rapid immune detection. Additional studies revealed that neosynthesis of antigen is required for direct presentation (Khan et al., 2001; Princiotta et al., 2003) further strengthening the DRiPs hypothesis. Newly synthesized, ubiquitinated proteins were observed to transiently accumulate in aggregates during DC maturation, further strengthening the DRiPs hypothesis that newly synthesized rapidly degraded proteins are the source of peptides for MHC class I presentation (Lelouard et al., 2004; Lelouard et al., 2002).

In vitro studies have demonstrated that many different forms of antigen can be transferred from the antigen donor cell to the APC in cross-priming. Exogenous RNA (Boczkowski et al., 1996) or DNA coding sequences (Cho et al., 2001), peptide (Carbone et al., 1988), MHC class I-peptide complexes (Dolan et al., 2006), HSP-peptide complexes (MacAry et al., 2004), apoptotic cells (Albert et al., 1998a; Albert et al., 1998b), necrotic cells (Gallucci et al., 1999), cell debris (Inaba et al., 1998), whole protein (Serna et al., 2003) and degradation intermediates (Serna et al., 2003) were all presented *in vitro*. When some of these substrates were tested *in vivo*, however, no CD8+ T cell response was stimulated (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996). The form of antigen transferred in the cross-priming pathway is currently unknown *in vivo*. Unlike direct priming, cross-priming can be separated from direct priming *in vivo* by using cells incapable of presenting antigen as antigen donor cells. Initial observations of cross-priming implied that the form of antigen transferred *in vivo* was particulate or aggregated protein (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996; Shi and Rock, 2002). However, Srivastava *et al.* observed that heat-shock proteins (HSP) isolated from tumors were immunogenic and stimulated tumor specific CD8+ T cells (Udono and Srivastava,

1993). It has been suggested that the immunogenic material in these studies was small peptides bound to the HSP (Srivastava et al., 1994; Suto and Srivastava, 1995; Udono and Srivastava, 1993). Several heat shock proteins were found to have such bound peptides including HSP70, HSP90 and gp96 (Berwin et al., 2002; Binder et al., 2001; Blachere et al., 1997; Singh-Jasuja et al., 2000). While these studies demonstrated the potential for whole protein or HSP-peptide complexes to be transferred in cross-presentation, the requirement for chaperones in the cross-priming pathway remains controversial (Baker-LePain et al., 2003; Nicchitta, 2003). Subsequent reports have demonstrated that peptides bound to gp96 were dissimilar in structure to MHC class I binding peptides (Demine and Walden, 2005) and that "purified" gp96 preparations actually contained immunogenically relevant levels of contaminating proteins (~473×10^6 molecules of contaminating protein/μg of gp96) (Reed et al., 2002), suggesting that HSP may actually function more as adjuvants than as the source of antigen transferred in cross-priming *in vivo*. Additionally, the lack of peptide accumulation within the cell *in vitro* fuels the controversy over chaperone-mediated transfer of antigen during cross-priming. Concentrations of intracellular, free peptide are extremely low, with the majority of these peptides present as part of complexes with MHC class I molecules or an undefined-protein which is not an HSP or a known chaperone protein (Falk et al., 1990; Malarkannan et al., 1995; Paz et al., 1999). Using fluorescent labeled peptides, Reits *et al.* demonstrated that microinjected peptides have high diffusion rates, are distributed mainly in the cytoplasm and nucleoplasm and do not accumulate in the ER (Reits et al., 2003). Peptide accumulation within the cell would have been expected if, as hypothesized, chaperones were able to bind to and retain peptides. This study also demonstrated that the half-life of peptides microinjected into the cell was less than 7 seconds, indicating that stable interactions with peptide binding proteins such as chaperones were extremely unlikely as such interactions would likely prevent peptide degradation by peptidases (Reits et al., 2003). Thus, although HSP have been demonstrated to induce cross-priming to tumor antigens *in vivo*, they

may not represent a physiologically relevant form of antigen for transfer *in vivo*. HSP-peptide complexes may still have a therapeutic role in cancer vaccination, but *in vivo* studies to determine the physiologically relevant form of antigen transferred in cross-priming would aid in the rationale design of vaccines. Therefore, studies examining the form of antigen transferred in cross-priming are needed to better characterize this pathway *in vivo*.

To enhance the activation of CD8+ T cells *in vivo* using the cross-priming pathway, it is necessary to determine what cellular functions are needed in the antigen donor cell to facilitate the donation of antigen. Cellular functions refers to cellular processes such as proteasomal degradation, protein synthesis and secretory function. The requirement for proteasomal degradation in the antigen donor cell will be addressed as part of the studies examining the form of antigen transferred *in vivo*. The requirement for protein synthesis in the antigen donor cell refers to both the neosynthesis of the antigen as well as to other proteins which may be required for the antigen to be transferred. The role of secretory function in the antigen donor cell is of particular interest as it is unknown how the antigen is transferred in the cross-priming pathway. Additionally, many viruses proposed as vaccine vectors abrogate specific cellular functions; in order to target an antigen to the cross-priming pathway it may be necessary to use vectors that permit certain functions in the antigen donor cell. Thus, in these studies we will not only examine the form of antigen transferred in cross-priming *in vivo* but will also determine which cellular functions are required in the antigen donor cell *in vivo*.

3.2 Results

3.2.1 Requirements to Study Cross-Priming *In Vivo*

In order to study cross-priming *in vivo*, it is necessary to use antigen donor cells that either do not express the same MHC class I molecule as the mouse they are injected into or that cannot generate MHC class I molecules owing to a defect in either MHC class I biosynthesis or protein folding (Hudrisier and Bongrand, 2002). Use of such antigen donor cells excludes the possibility of direct priming from these cells, allowing the cross-priming pathway to be isolated *in vivo*. The majority of the experiments examining cross-priming *in vivo* described here will make use of antigen donor cells that do not express MHC class I on the cell surface due to a targeted deletion in the gene encoding β_2-microglobulin subunit of MHC class I (Norbury et al., 2004). However, some experiments will make use of human cell lines (which do not express MHC class I molecules recognized by the mouse) or mouse cells that express a mutated form of MHC class I not recognized by mouse T cell receptors (Clarke et al., 2000).

When infected antigen donor cells are used to study cross-priming *in vivo*, it is necessary to prevent infection of the host animal into which they are injected. Infection of the host animal by the viruses used to infect the antigen donor cells would allow the cells of the host animal to express the antigen, thus permitting direct priming of that antigen. Inactivation of the viruses used to infect the antigen donor cells prior to the injection of infected antigen donor cells ensures that the observed CD8+ T cell responses are based on cross-primed antigen rather than direct priming. In experiments using vaccinia virus (VV) infected antigen donor cells, viral spread is prevented by treating the cells with psoralen and ultraviolet (UV)-irradiation (Tsung et al., 1996) or by paraformaldehyde fixation (Hulskotte et al., 1997) of the infected cells (Figure 3.1).

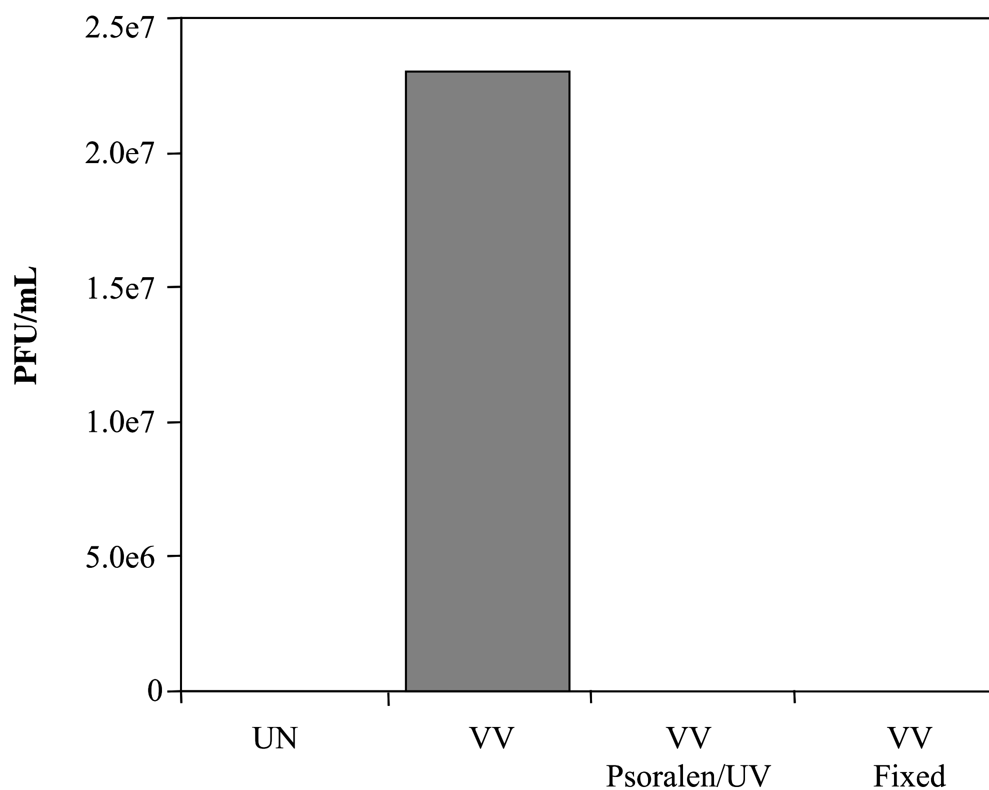

Figure 3.1 Treatment of VV-infected cells with psoralen/UV or paraformaldehyde fixation prevents viral replication.

β_2m_{neg} cells were mock infected (UN) or infected with VV (VV) for 4 hours prior to psoralen/UV treatment (Psoralen/UV) or paraformaldehyde fixation (fixed) as indicated. After treatment cells were processed according to the protocol for harvesting VV. Viral replication was determined by plaque assay. Data are expressed as plaque forming units per mL and are representative of 3 experiments.

However, in experiments using influenza A virus (IAV), infection of the host animal was prevented by mixing cells with IAV neutralizing antibody prior to injection. Theoretically, the addition of a neutralizing antibody could enhance priming through antibody-mediated uptake of antigen. To control for this, the IAV experiments always included a virus plus neutralizing antibody control to demonstrate that antibody-mediated uptake of antigen did not contribute to priming.

3.2.2 Nature of the Cross-Primed Antigen *In Vivo*

Determining the nature of the antigen transferred from the antigen donor cell to the antigen presenting cell *in vivo* requires the use of multiple recombinant vaccinia virus (rVV) constructs expressing various forms of antigens. The rVV constructs used in these experiments are depicted in Figure 3.2. For the majority of the experiments, different forms of the model antigen ovalbumin (OVA) were used. Each of these forms contained the minimal OVA antigenic epitope, $OVA_{257-264}$, that is presented on K^b and recognized by CD8+ T cells.

3.2.2.1 Can minimal antigenic peptide be a substrate for cross-priming?

According to the hypothesis that chaperone-bound peptides are the substrate for cross-priming *in vivo*, peptides generated in the antigen donor cell could be bound to chaperones and transferred in cross-priming *in vivo*. Thus, pre-processed peptides were examined to determine whether pre-processed peptides could serve as the antigenic substrate in cross-priming. Under the peptide pulsing conditions used in this experiment, the synthetic $OVA_{257-264}$ peptide would have free access to the endoplasmic reticulum via a vesicular transport route (Day et al., 1997), permitting potential association with some of the molecular chaperones hypothesized to be

Figure 3.2

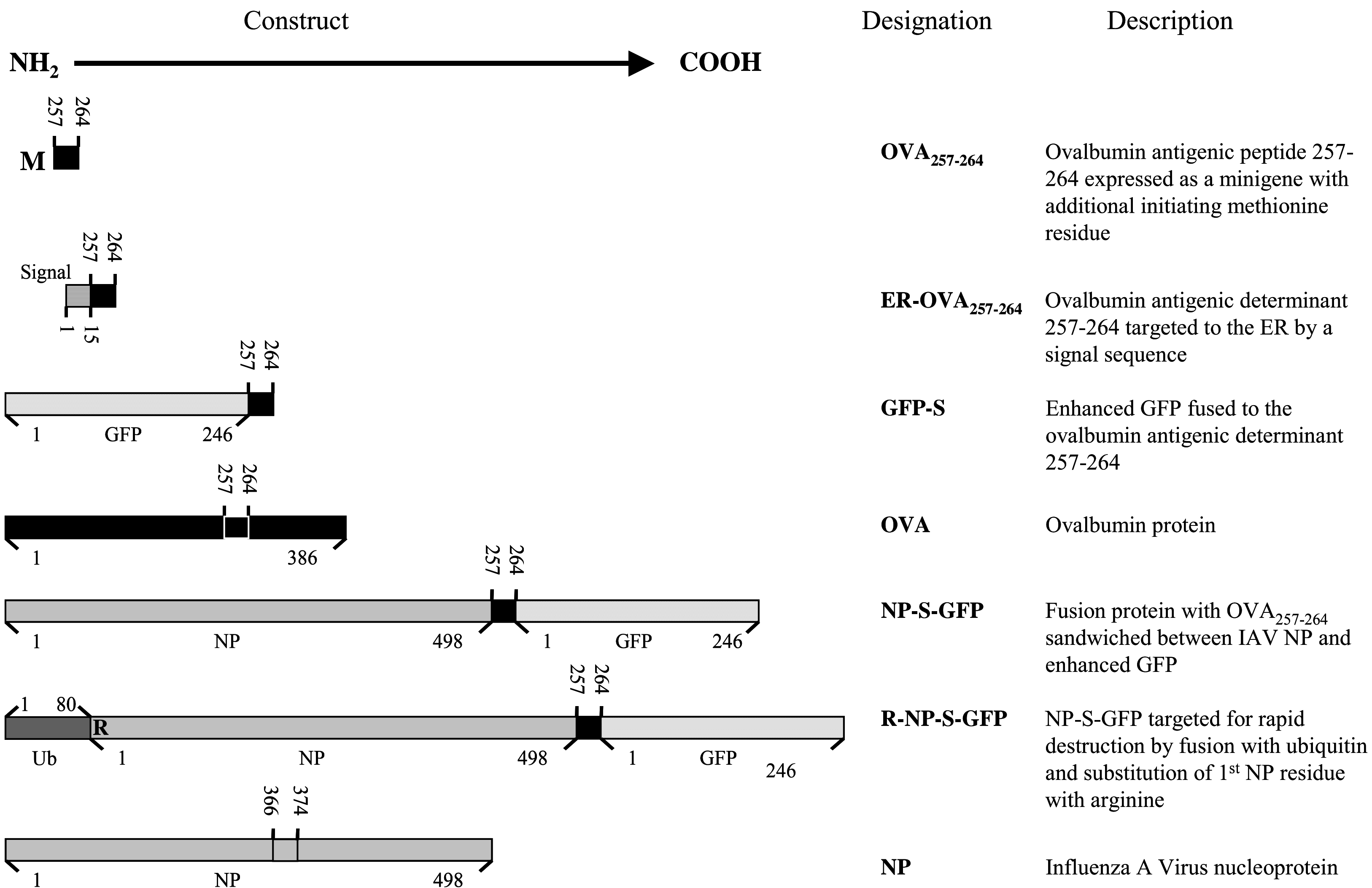

Figure 3.2 Schematic of expression vector– encoded gene products used to study cross-priming. The gene constructs utilized in this study are schematically diagrammed.

involved in cross-priming. Synthetic $OVA_{257-264}$ was loaded onto splenocytes derived from mice with the bm1 mutation of K^b (Clarke et al., 2000). Seven amino acid substitutions distinguish K^{bm1} from K^b. T cell activation is inhibited by the amino acid substitution of tyrosine for arginine at position 155 in K^{bm1} (Clarke et al., 2000; Rudolph et al., 2001). Using FITC-conjugated $OVA_{257-264}$, it was determined that $OVA_{257-264}$ can bind to K^{bm1} with an affinity similar to that of K^b (Figure 3.3A). Fluorescent-labeled OT-I T cells were adoptively transferred into B6 mice that were subsequently immunized with $OVA_{257-264}$ loaded splenocytes from either wild-type (K^b) or K^{bm1} mice. Proliferation of the adoptively transferred OT-I cells was then used as a measure of cross-priming. Splenocytes from wild-type mice (which are able to directly prime the OT-I T cells) pulsed with as little as 10^{-9}M peptide were able to induce OT-I proliferation but peptide pulsed K^{bm1} splenocytes failed to elicit measurable OT-I proliferation even at 3 logs higher than the highest peptide concentration used to elicit a response to wild-type (Figure 3.3B). The failure of peptide pulsed K^{bm1} splenocytes to activate CD8+ T cells suggests that neither MHC class I molecules or chaperones are able to mediate cross-priming under these conditions.

To examine whether peptide can be a substrate for cross-priming under conditions of infection, we infected β_2-microglobulin negative antigen donor cells with rVV constructs expressing cytosolic ($OVA_{257-264}$) or ER-targeted $OVA_{257-264}$ (ER-$OVA_{257-264}$) minigene products (Figure 3.2). An $OVA_{257-264}$ minigene product expressed as a fluorescent chimeric protein (NP-S-GFP) was used as a positive control (Figure 3.2). When B6 mice were infected with one of the rVVs mentioned above, permitting direct priming, IFN-γ expression was observed in OVA-specific CD8+ T cells in response to each virus expressing OVA (Figure 3.4A). No IFN-γ expression was observed in mice that received an rVV that did not express OVA (Figure 3.4A). In contrast, immunization of B6 mice with P815 (MHC mismatched, H-2^d) cells infected with either the cytosolic minigene or the ER targeted minigene construct failed to induce IFN-γ producing CD8+

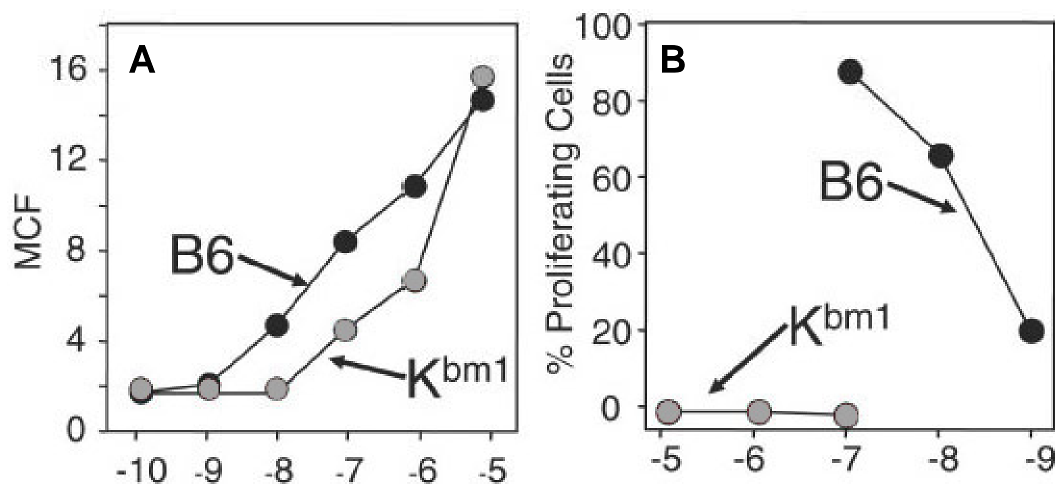

Figure 3.3 Cross-priming is not mediated by exogenous peptides.

We measured binding of $OVA_{257-264}$ to splenocytes derived from B6 or K^{bm1} mice by flow cytometry using a fluorescent version of the peptide that mimics the binding of the unmodified peptide (A). An additional experiment yielded similar results. MCF, mean channel fluorescence. Splenocytes from B6 or K^{bm1} mice pulsed with synthetic $OVA_{257-264}$ peptide at the indicated concentrations were injected into B6 mice into which fluorescent OT-I TCR transgenic CD8+ T cells were adoptively transferred 3 hours previously (B). The percentage of dividing OT-I cells was determined flow cytometrically by decreased cellular fluorescence. This experiment was repeated twice with similar results. Data for figure courtesy of the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda Maryland.

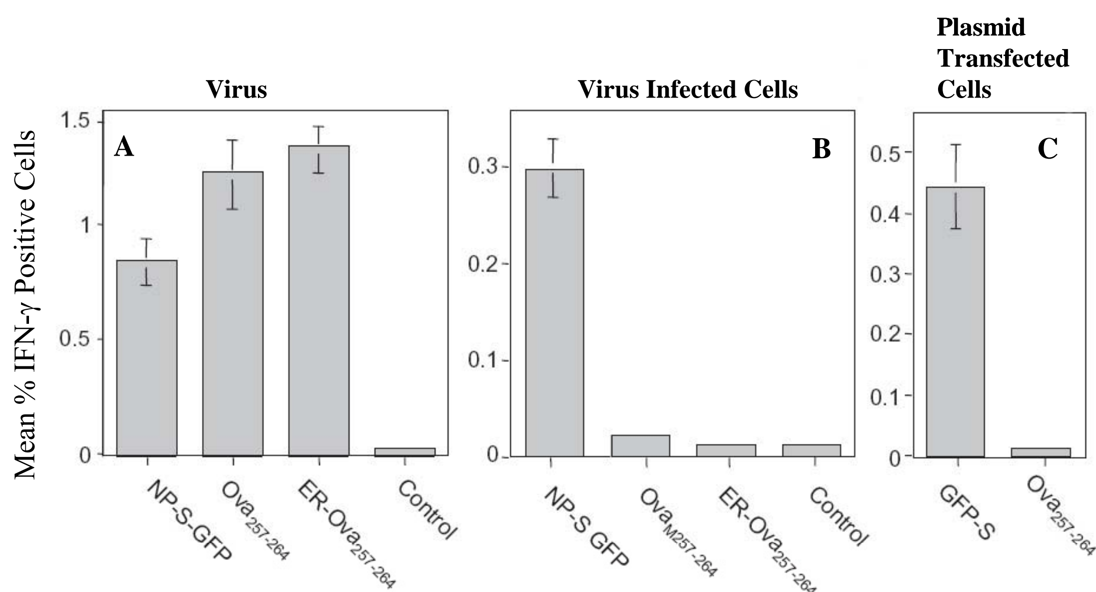

Figure 3.4 Cross-priming is not mediated by endogenous peptides.

B6 mice were immunized with the rVV expressing the indicated gene product (A) or with P815 cells infected for 12 hours with rVVs expressing the gene product indicated (B). Six days later, numbers of responding $OVA_{257\text{-}264}$-specific CD8+ T cells present in spleens were determined by their expression of IFN-γ. Data represent averages from three mice for each group; errors bars show ±SEM. The figure is representative of nine additional experiments performed with P815. After 293 cells were transfected 24 hours earlier with plasmids encoding the indicted gene product, they were introduced into B6 mice, and the responding CD8+ T cells enumerated by expression of IFN-γ (C). Averages from four mice for each group with ±SEM are shown. An additional experiment yielded similar results. by decreased cellular fluorescence. This experiment was repeated twice with similar results. Data for figure courtesy of the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda Maryland.

T cells (Figure 3.4B). Cross-priming was detected from the control rVV expressing the $OVA_{257-264}$ as part of a fusion protein (Figure 3.4B), implying that the protein, rather than the minimal antigenic epitope, had the ability to be cross-primed. The ability of the fusion protein to stimulate cross-priming was not due to an increased expression level of the fusion protein as similar amounts of K^b-$OVA_{257-264}$ complexes were produced from both the $OVA_{257-264}$ minigene and the fusion protein (Figure 3.5). As mentioned above, in experiments using rVV-infected antigen donor cells, UV irradiation is used just prior to immunization to prevent transfer of infectious virus from the antigen donor cells. However, because UV irradiation inhibits protein synthesis and minigene products are rapidly degraded within the cells (Anton et al., 1997), it remained possible that uninfected cells actively synthesizing minigene products could function in cross-priming.

To investigate this possibility, we used 293 (human) cell lines that either express cytosolic $OVA_{257-264}$ alone or cytosolic $OVA_{257-264}$ as a chimeric fusion protein (GFP-S) (Figure 3.2) from transfected plasmids. Mice immunized with these cell lines induced IFN-γ production from CD8+ T cells only in response to the chimeric protein (Figure 3.4C). No IFN-γ production was observed from CD8+ T cells in response to the donor cells expressing cytosolic $OVA_{257-264}$ only (Figure 3.4C), indicating that minimal antigenic peptides did not cross-prime T cells *in vivo*.

3.2.2.2 Is rapidly degraded protein a substrate for cross-priming?

The data from the experiments shown in Figure 3.4 indicate that cells expressing stable proteins are able to induce cross-priming while cells expressing minimal antigenic peptides are not. Proteasomes, however, do not typically generate minimal peptides (Shastri et al., 2002; York et al., 2002). Thus, to investigate the possibility that cells producing “natural” proteasome products would be able to induce cross-priming, we infected the P815 (MHC mismatched, H-2^d)

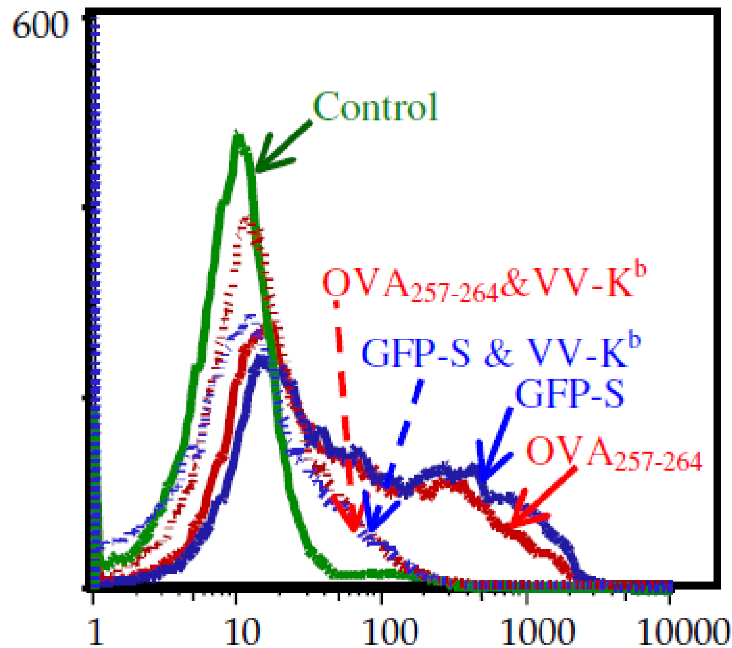

Figure 3.5 Expression of K^b-$OVA_{257\text{-}264}$ complexes in transfected 293 cells.

Solid lines indicate 293- K^bC2 cells transfected with plasmids expressing GFP-$OVA_{257\text{-}264}$ or $OVA_{257\text{-}264}$ and assayed 24 hours later for cell surface expression of K^b-$OVA_{257\text{-}264}$ complexes by flow cytometry using the 25-D1.16 monoclonal antibody conjugated to Alexafluor 647. Dotted lines indicate 293 cells similarly transfected, but were infected with rVV- K^b 12 hours p.i. and then assayed as above for K^b-$OVA_{257\text{-}264}$ complex expression 4 hours later. by decreased cellular fluorescence. This experiment was repeated twice with similar results. Data for figure courtesy of the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda Maryland.

cell line with an rVV construct expressing a rapidly degraded chimeric protein (R-NP-S-GFP) (Figure 3.2). This virus was highly immunogenic when used to infect mice (Figure 3.6A). Cells expressing this rapidly degraded chimeric protein, however, were unable to cross-prime unless they were treated with the proteasome inhibitor lactacystin to prevent its degradation (Figure 3.6B). This finding demonstrates that proteasome products were not able to cross-prime, once again implying that stable proteins are the form of antigen transferred in cross-priming *in vivo*.

3.2.3 Characterization of the Requirements of the Antigen Donor Cell

While the molecular interactions, proteolytic degradation pathways and vesicular trafficking events required for direct presentation of antigen have been extensively studied *in vitro*, the functional requirements in the antigen donor cell of the cross-priming pathway are unknown. First, we sought to complete our studies on the form of antigen transferred in cross-priming *in vivo* by further examining the requirement for proteasomal processing. Then, we will examine whether neosynthesis of antigen, protein synthesis of cellular factors and secretory function are required in the antigen donor cell in order for antigen to be available for donation to the cross-priming pathway.

3.2.3.1 Is proteasomal degradation required in the antigen donor cell **in vivo***?*

The studies described above indicated that the form of antigen transferred is a protein, rather than minimal antigenic peptides or proteasomal intermediates. Thus, determining the requirement for proteasomal degradation in the antigen donor cell will confirm whether the form of antigen transferred *in vivo* is protein or degradation intermediates.

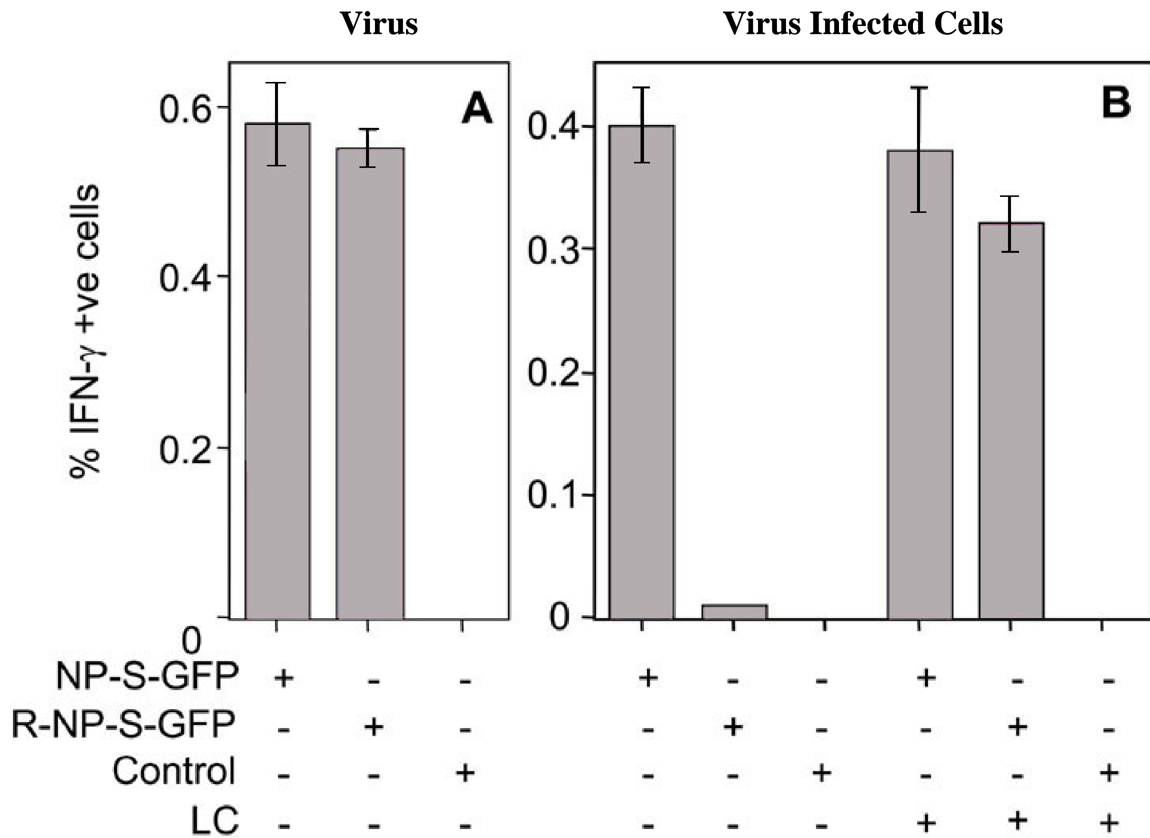

Figure 3.6 Rapidly degraded proteins are not substrates for cross-priming.

Mice were immunized with rVV expressing the protein indicated (A) or P815 cells infected for 12 hours with rVVs expressing the gene product indicated (B). Six days after immunization, the percent of $OVA_{257-264}$-specific CD8+ T cells activated to produce IFN-γ was determined. Where indicated, 20μM lactacystin was added to cells 1 hour after addition of virus and was maintained throughout the 12-hour infection. Averages from three mice for each group. Panels are representative of three experiments.

3.2.3.1.1 Characterization of the β_2-microglobulin negative cell response to the proteasome inhibitor lactacystin

To examine the requirements for proteolytic degradation in the antigen donor cell, we first had to demonstrate that proteasome degradation could be inhibited in the β_2-microglobulin negative cells using lactacystin, an irreversible proteasome inhibitor (Fenteany et al., 1994), over the time course to be used in our *in vivo* studies. Thus, β_2-microglobulin negative cells were treated with (or without) lactacystin, for 1 hour prior to infection and for an additional 4 hours after infection or mock infection. Cells were infected with rVV expressing either a stable or rapidly degraded form of a fusion protein of the minimal antigenic epitope of OVA, $OVA_{257-264}$, sandwiched between the influenza A virus (IAV) nuclear protein (NP) and GFP (see Figure 3.2 for rVV construct designations). Lactacystin treated and untreated β_2-microglobulin negative cells were then washed, psoralen UV-irradiation treated and incubated for up to an additional 12 hours in the absence of lactacystin. Protein expression was monitored by GFP fluorescence as determined by flow cytometry with fluorescence inversely proportional to degradation. Protein expression showed that the degradation of the normally rapidly degraded construct Ub-R-NP-S-GFP was inhibited for at least 12 hours after lactacystin was withdrawn (Figure 3.7).

3.2.3.1.2 Is proteasomal processing required in the antigen donor cell?

The effects of the proteasomal inhibitor lactacystin were irreversible on β_2-microglobulin negative cells over the desired time course. Thus, the requirement for proteasomal processing in the antigen donor cell could now be probed *in vivo*. β_2-microglobulin negative cells were treated (or untreated) as before with lactacystin 1 hour prior to and 4 hours following infection with an rVV expressing OVA. Infected cells were then introduced into B6 mice (haplotype H-2^b) that had previously received transgenic CD8+ T cells from OT-I mice. Eighteen hours after

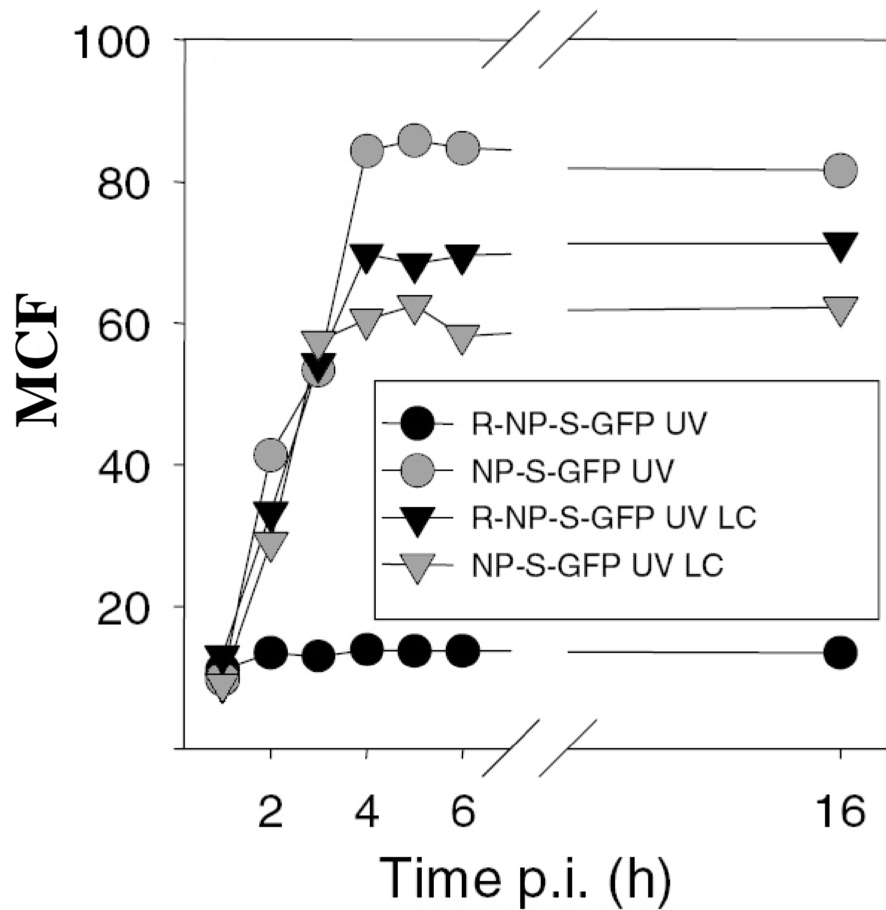

Figure 3.7 Characterization of β_2-microglobulin negative cells.

$\beta_2 m_{neg}$ cells were treated with (or without) 20mM lactacystin for 1 hour, infected with the rVV indicated and treated with (or without) 20mM lactacystin for an additional 4 hours prior to washing, psoralen UV-irradiation and incubation for up to an additional 12 hours. Protein expression was monitored by GFP fluorescence and was determined by flow cytometry. Data are expressed as mean channel fluorescence (MCF) vs. time post-infection. Panel is representative of three experiments.

immunization, the activation of the transferred OT-I cells was measured by increased cell surface expression of the activation marker CD69. Rather than inhibiting T cell activation, inhibition of proteasomal degradation by lactacystin had either no effect (6 experiments) or an enhanced effect in (3 experiments) on T cell activation (Figure 3.8A). This corroborated our results that degradation intermediates are not the form of antigen transferred during *in vivo* cross-priming.

To test whether the effect of proteasomal inhibition was the same for other antigens, we immunized B6 mice with β_2-microglobulin negative cells treated (or untreated) as in the above experiment and infected with influenza A virus. To prevent direct infection of mice with IAV released from the infected antigen donor cells, we co-injected the antigen donor cells with a monoclonal antibody (α-HA) that neutralizes IAV infectivity and prevents induction of CD8+ T cells responses by infectious IAV (Figure 3.8B). CD8+ T cell responses were measured 6 days after immunization using tetrameric D^b molecules complexed to an immunogenic peptide, amino acid residues 366-374, from the IAV nucleoprotein (NP). The use of the influenza system also served to control for the fact that OVA is a secreted protein and would be more readily available as a substrate for cross-priming than a non-secreted protein. Once again, inhibition of proteasomal degradation in the antigen donor cells had no effect on cross-priming, implying that proteasomal degradation in the antigen donor cell was not necessary for cross-priming to occur (Figure 3.8C). This finding, taken with the findings in Figure 3.6, emphasize that proteasomal degradation is not required in the antigen donor cell for cross-priming. This implies that stable proteins, rather than peptides or degradation intermediates, are the form of antigen transferred from the antigen donor cell *in vivo*.

3.2.3.2 Requirement for neosynthesis of antigen

Neosynthesis of antigen has been demonstrated to be a requirement for the direct priming

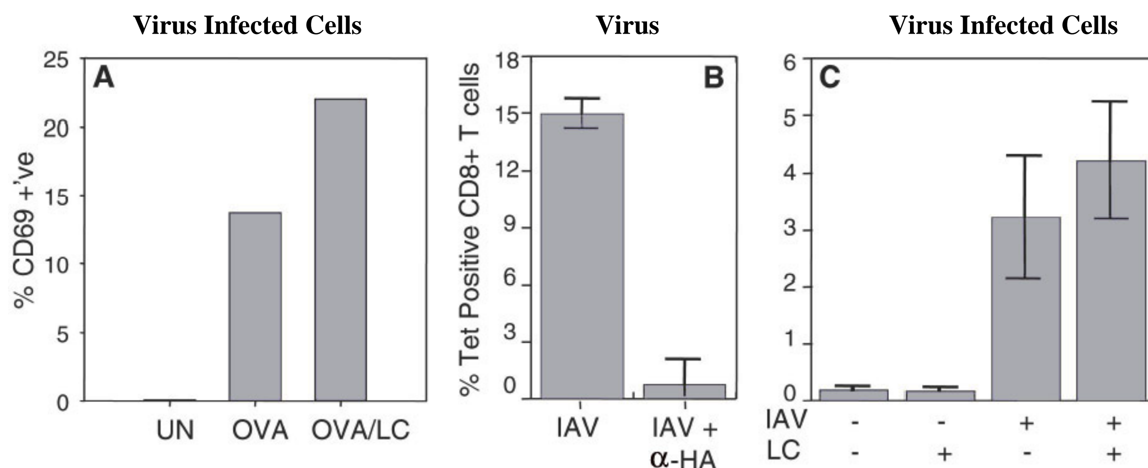

Figure 3.8 Cross-priming does not require proteasome activity in virus-infected cells.

(A) Uninfected β_2m_{neg} cells (UN) or cells infected with VV-ovalbumin (OVA) were injected 4 hours after infection into mice that had received OT-I TCR CD8+ T cells 3 hours previously. "OVA/LC" cells were treated with the irreversible proteasome inhibitor lactacystin, which was started 1 hour before infection. Sixteen hours after immunization, activation of CD8+, $V_{\alpha 2}$+ OT-I T cells was determined via measurement of increased expression of CD69 by flow cytometry. Data are expressed as the percentage of CD69 highly expressing cells. These data are representative of nine consecutive experiments in which immunogenicity was either enhanced (3 times) or remained unaffected by lactacystin treatment (6 times). (B) Mice were immunized intraperitoneally (i.p.) with IAV in the presence or absence of a neutralizing monoclonal antibody (mAb) against hemagglutinin (α-HA). Six days later, influenza virus–specific CD8+ T cells present in spleens were enumerated by flow cytometry using D^b-$NP_{366\text{-}374}$ molecules tetramerized by binding to fluorescent strepavidin. The percentage of CD8+ T cells that specifically bind tetramers is shown. (C) Uninfected β_2m_{neg} cells or β_2m_{neg} cells infected with IAV for 5 hours were injected i.p. in the presence of a neutralizing mAb against HA, and CD8+ T cells were enumerated as in (B). Where indicated, cells were treated with lactacystin (LC) from 1 hour before infection. The data shown represent the mean ±SEM of duplicate mice and are representative of four similar experiments.

pathway (Khan et al., 2001; Princiotta et al., 2003). To address whether neosynthesis of proteins was required for donation of antigen during cross-priming *in vivo* it was necessary to have a system in which both the initiation and cessation of antigen synthesis could be controlled. In order to achieve this we expressed β-galactosidase (β gal), a model antigen, driven by an inducible promoter. β gal was expressed in 293A cells transfected with the tet repressor, which produce undetectable amounts of protein in the absence of tetracycline. A similar study examining the requirement for neosynthesis of antigen for direct presentation utilized nucleoprotein (NP) from LCMV as an antigen (Khan et al., 2001). In contrast to NP, our model antigen, β gal, is an enzyme that can be detected at very low quantities while relatively large quantities of LCMV NP are needed for detection via Western blot. We super-transfected 293A cells already expressing the tetracycline repressor with the β gal expressing plasmid and characterized expression as follows.

3.2.3.2.1 Selection of β gal expressing clones

Transfected cells were treated with tetracycline and stained with the β gal substrate X gal to visualize expression of the transgene. A positive staining colony, designated L4.2 (Figure 3.9), was selected and used for all subsequent experiments. Greater than 90% of L4.2 cells expressed β gal after 4 hours of tetracycline induction and less than 0.1% of the cells expressed β gal in the absence of tetracycline treatment (Figure 3.10A).

3.2.3.2.2 Characterization of β gal expression upon tetracycline induction

L4.2 cells were treated with tetracycline for 4 hours and then cultured in the absence of tetracycline to determine both the time point at which new synthesis of β gal ceased (Figure 3.10B) and the amount of time that β gal lingered in the cells after withdrawal of treatment (Figure 3.10C). β gal production was assayed in bulk L4.2 cultures using the chromogenic β gal

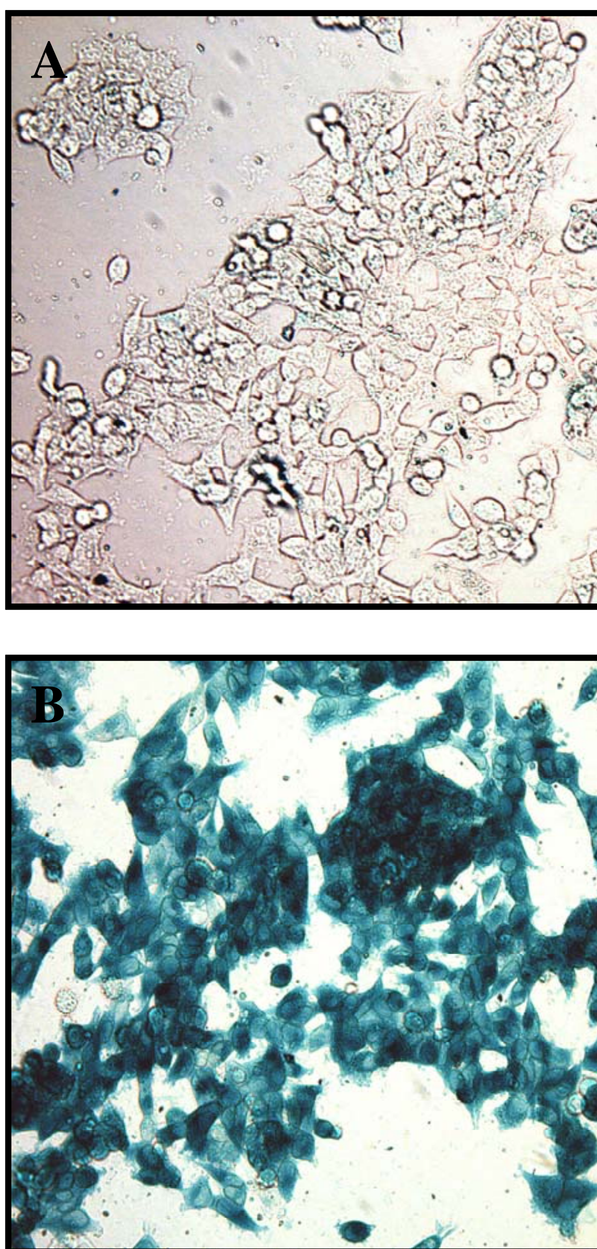

Figure 3.9 L4.2 cells express β gal upon tetracycline induction.

L4.2 cells were either untreated (A) or treated (B) with 1μg/mL tetracycline for 4 hours and then stained with the β gal substrate X gal. 24 hours later photos were taken using a deconvolution microscope. Representative fields are shown.

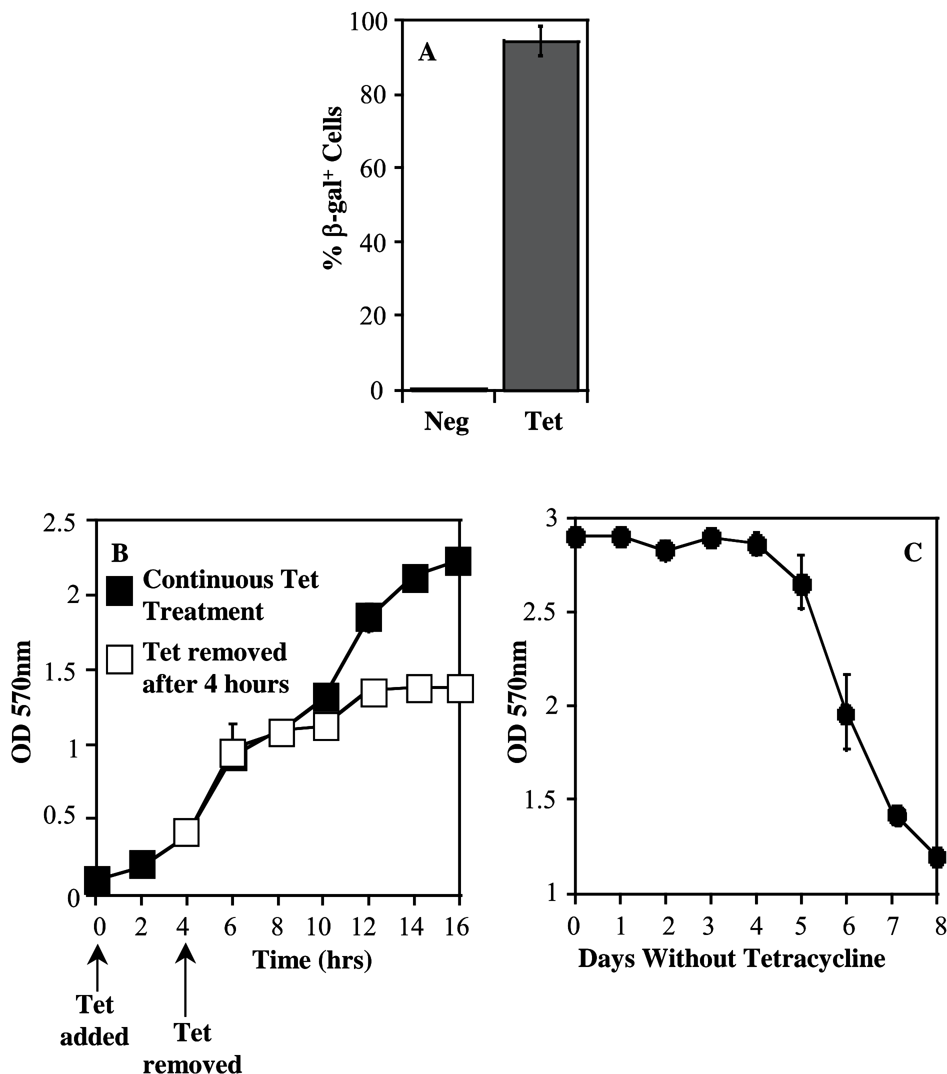

Figure 3.10 Characterization of β gal production in L4.2 Cells.

L4.2 cells were treated with 1μg/mL tetracycline for 4 hr and the percentage of cells producing β gal (A), the time point after tetracycline withdrawal at which new synthesis of β gal ceases (B) and the length of time β gal persists in the cell after treatment is withdrawn (C), was measured. (B) Cells were cultured in the presence of tetracycline for either 4 hr (open squares) or for the entire duration of the experiment (closed squares). β gal was quantitated by X gal (A) or CPRG staining (B, C). Error bars indicate the standard error of the mean. Each panel represents 3-5 experiments.

substrate CPRG. As shown in Figure 3.10B, new protein synthesis of β gal ceased 8 hours after the withdrawal of tetracycline, as after this time point intracellular levels of β gal did not increase. The failure to increase β gal levels is likely a result of a shut down of antigen synthesis, but it could also be the result of decreased antigen production accompanied by proteolysis of enzymatically active β gal. β gal generally has a half-life in the order of days (Bachmair et al., 1986; Smith et al., 1995). To determine whether antigen degradation could account for the reduced β gal levels in L4.2 cells 8 hours after withdrawal of tetracycline, we examined the reduction in levels of β gal over a number of days. Levels of β gal remained constant in L4.2 cells for an additional 5 days before a decrease could be observed, indicating that degradation is not responsible for the flattening of the β gal production early after tetracycline withdrawal. Indeed, β gal is still detectable by CPRG assay 8 days after tetracycline has been withdrawn from the culture.

3.2.3.2.3 Requirement of neosynthesis of antigen for direct presentation

In order to measure direct presentation of antigen it was necessary to express an MHC Class I molecule in the L4.2 cells. L4.2 cells were retrovirally transduced to express the murine MHC Class I molecule H2-K^b (Figure 3.11A) to generate L4.2mK^b. L4.2mK^b cells were also tested to verify that the kinetics of β gal expression after tetracycline induction remained unchanged (Figure 3.11B).

The requirement for neosynthesis of antigen for direct presentation was examined by measuring activation of a T cell line cultured from Bg1.SJL splenocytes by L4.2mK^b cells. Bg1.SJL mice express a transgenic T cell receptor specific for amino acid residues 96-103 of β gal. Cultured Bg1.SJL T cells are not considered naïve T cells as they are stimulated with a cell

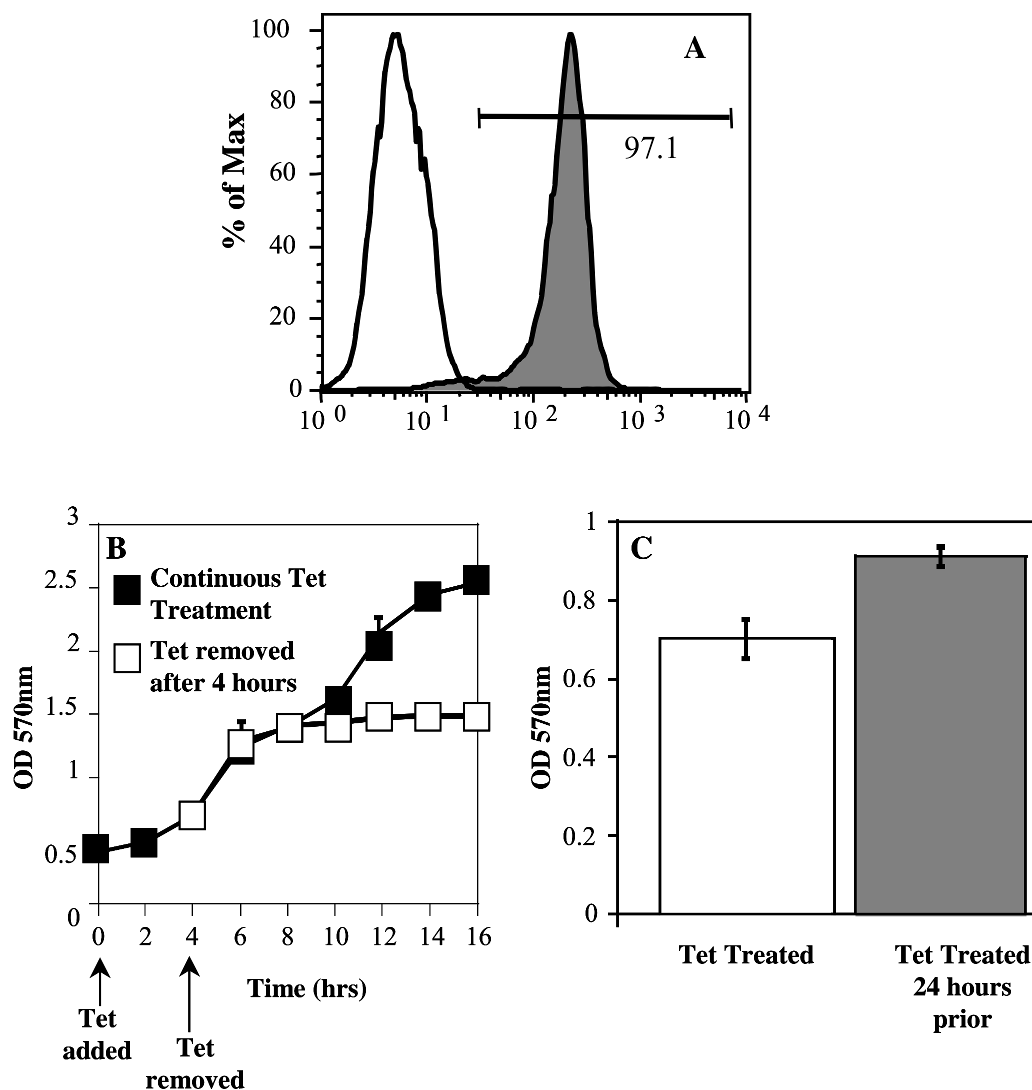

Figure 3.11 Characterization of L4.2mK^b Cells.

(A) L4.2mK^b cells were evaluated for their ability to express K^b after flow cytometric sorting of K^b-expressing cells by MoFlo. Filled peak indicates L4.2mK^b cells, unfilled peak indicates L4.2 cells. (B) The kinetics of β gal production was measured by CPRG staining in L4.2mK^b cells which were treated with 1μg/mL tetracycline (closed boxes) or treated with 1μg/mL tetracycline for 4 hours prior to culture in the absence of tetracycline (open boxes). (C) The amount of β gal produced in L4.2mK^b cells treated with 1μg/mL tetracycline 24 hours prior to (closed box) or immediately prior to (open box) CPRG staining. Panels A and C are representative of 3 experiments while panel B represents a single experiment. Error bars indicate the standard error of the mean.

line, stably expressing β-gal and provided with IL-2 in the culture media. L4.2mK^b cells in which β gal production had recently been induced by tetracycline treatment, or L4.2mK^b that had been treated with tetracycline for 4 hours but then cultured in the absence of tetracycline for 24 hours, were used to stimulate the Bg1.SJL T cell line. L4.2mK^b that were no longer synthesizing β gal (24 hours after tetracycline treatment) had equivalent levels of intracellular β gal to those recently treated with tetracycline (Figure 3.11C). Activation of T cells was measured by induction of IFN-γ production. L4.2mK^b cells that were no longer synthesizing β gal triggered few Bg1.SJL cells to produce IFN-γ, with activation levels being marginally above background (Figure 3.12A). In contrast, L4.2mK^b cells that had recently been treated with tetracycline, and so were still synthesizing β gal, were able to stimulate much greater numbers of Bg1.SJL T cells to produce IFN-γ (Figure 3.12A). Thus, neosythesis of antigen greatly enhances the efficiency of direct presentation. Similar results were observed when L4.2 cells infected with rVV-K^b for 2 hours to induce expression of H2-K^b were used (Figure 3.12C) to stimulate Bg1.SJL T cells. β gal-specific T cells were not activated under any conditions in the absence of mK^b expression (Figure 3.12B).

3.2.3.2.4 Requirement for neosynthesis of antigen in cross-priming **in vivo**

As L4.2 cells do not present β $gal_{96\text{-}103}$ in the absence of mK^b expression they are ideally suited for use as antigen donor cells with which to study the requirement for new protein synthesis during cross-priming *in vivo*. T cells were harvested from Bg1.SJL mice, labeled with CFDA-SE, and adoptively transferred into wild-type C57BL/6 recipient mice. These mice were then immunized with L4.2 that had either recently been treated with tetracycline or treated with tetracycline 24 hours previously. Mice immunized with L4.2 cells that had not been treated with tetracycline were included as a negative control group. Seventy-two hours after immunization, Bg1.SJL CD8 T cell proliferation was measured by dilution of CFDA-SE fluorescence. Comparable Bg1 proliferation was seen from L4.2 cells treated with tetracycline, whether it was

Figure 3.12

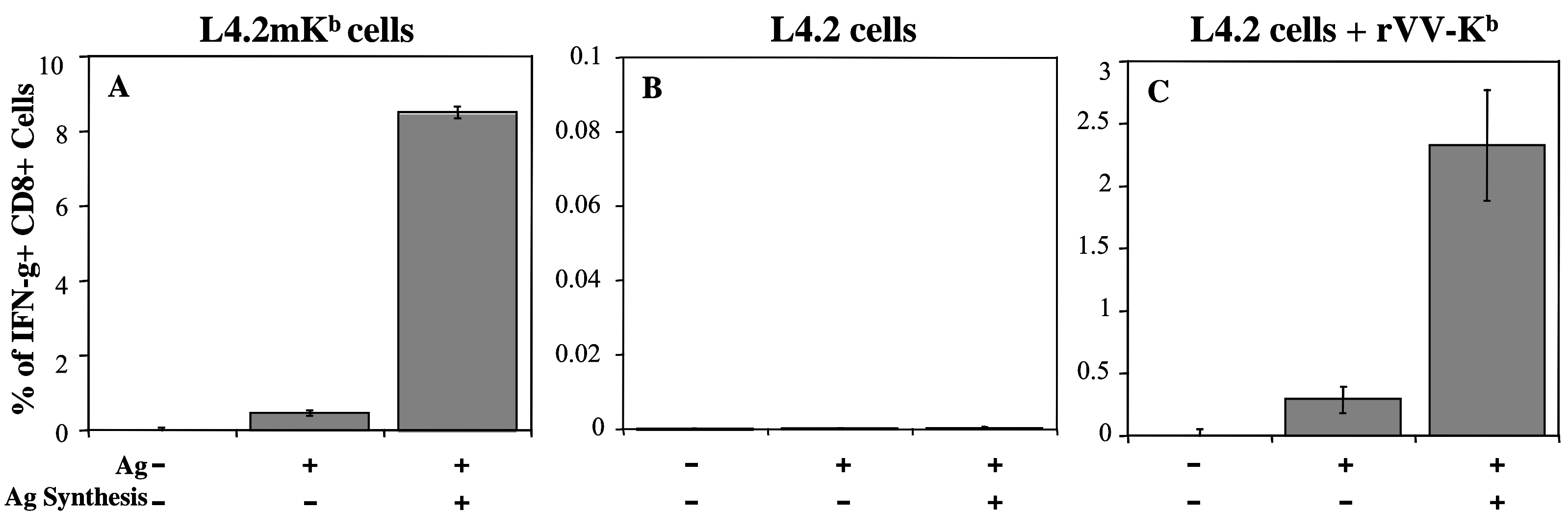

Figure 3.12 Antigen synthesis is required for direct presentation.

(A) Untreated L4.2mK^b cells or 1μg/mL tetracycline-treated cells; tetracycline was withdrawn immediately prior to or 24 hr prior to co-culture with cultured β gal$_{96-103}$-specific T cells. CD8+ cells were then evaluated for IFN-γ production. (B) Same as (A) except that L4.2 cells were used. (C) Same as (A) except that L4.2 cells infected with rVV-K^b 2 hr prior to co-culture with T cells were used. Each panel is representative of 3 experiments. Error bars indicate the standard error of the mean.

withdrawn just prior to injection or 24 hours prior to injection (Figure 3.13B, C). No proliferation was observed in response to untreated L4.2 cells (Figure 3.13A). Thus, in contrast to direct presentation, where active synthesis of protein was required for efficient presentation (Figure 3.12) new synthesis of antigen was not required for cross-priming *in vivo* (Figure 3.13).

3.2.3.3 Requirement for cellular protein synthesis

We have demonstrated that antigen synthesis is not required for donation of antigen during cross-priming *in vivo*. However, it is possible that synthesis of other cellular proteins is required for donation of antigen, perhaps via association of newly synthesized cellular proteins with antigen. Thus, we sought to examine the requirement for cellular protein synthesis in both direct and cross presentation.

3.2.3.3.1 Requirement for cellular protein synthesis for direct presentation

To examine whether association with newly synthesized cellular factors is required for antigen presentation, it is necessary to introduce antigen following the inhibition of protein synthesis. However, initial experiments with electroporating OVA into $H2^b$ WT3 cells revealed that the concentration of OVA titrated for *in vivo* experiments, 1mg/mL, was insufficient to activate an OVA-specific T cell hybridoma *in vitro*. *In vitro* titrations revealed that electroporation of 10mg/mL OVA was necessary for WT3 cells to activate an OVA-specific T cell hybridoma. To address the requirement for cellular protein synthesis in direct presentation, $H2^b$ WT3 cells were treated with the protein synthesis inhibitor cycloheximide for 1 hour, then electroporated the cells with 10mg/mL OVA. OVA used in these experiments was free of contaminating peptides as it was unable to activate an OVA-specific T cell hybridoma when co-cultured with antigen presenting cells that were fixed (to prevent processing of the protein) prior

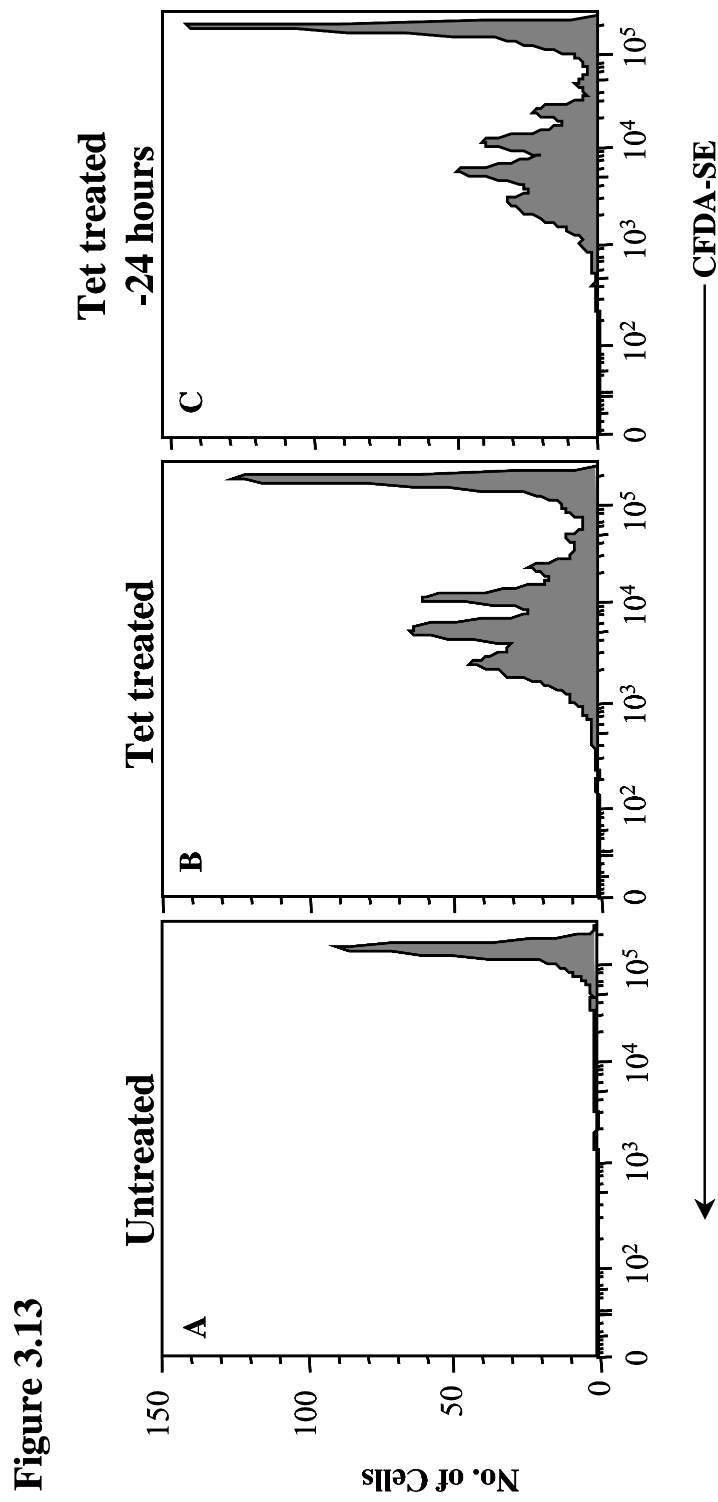
Figure 3.13
Untreated
Tet treated
Tet treated
-24 hours
A
B
C
No. of Cells
150
100
50
0
0
10²
10³
10⁴
10⁵
CFDA-SE

Figure 3.13 Antigen synthesis is not required for antigen donation during cross-priming *in vivo*.

B6 mice that had received an adoptive transfer of CFDA-SE-labeled Bg1.SJL cells were immunized with untreated L4.2 cells (A), or 1μg/mL tetracycline-treated L4.2 cells (B, C) where tetracycline treatment was withdrawn immediately before injection (B) or 24 hours before injection (C). Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Each panel is representative of 4 experiments.

to addition of OVA. Cycloheximide is readily reversible, so 5 hours after electroporation cells were fixed to prevent resumption of cellular protein synthesis. Removal of the inhibitor was necessary in order to assay antigen presentation by measuring activation of the K^b-restricted $OVA_{257\text{-}264}$-specific B3Z hybridoma. B3Z produce β gal upon activation, therefore B3Z were co-cultured with WT3 overnight and activation measured by staining with the β gal substrate X-Gal. Electroporated and fixed WT3 efficiently activated B3Z, but treatment of WT3 with cycloheximide completely ablated B3Z activation (Figure 3.14A). Similar results were observed after treatment of WT3 with the irreversible protein synthesis inhibitor emetine (Figure 3.14C). Fixed WT3 that were either treated with cycloheximide or untreated efficiently presented exogenous peptide to B3Z (Figure 3.14). To rule out the possibility that the inhibitory effect of cycloheximide was a non-specific toxic effect we treated cells for 5 hours as above, then removed the drug without fixing the APC. After removing cycloheximide, WT3 recovered their ability to present peptide produced from electroporated OVA (Figure 3.14B). Thus, specific blockade of protein synthesis prevented direct presentation.

3.2.3.3.2 Requirement for cellular protein synthesis for cross-priming **in vivo**

To assess the role for cellular protein synthesis in donation of antigen during cross-priming we treated murine H-2^b cells lacking β_2-microglobulin (β_2-microglobulin negative cells) with the irreversible protein synthesis inhibitor emetine. Cyclohexamide was not used *in vivo* because it is readily reversible. After treatment with emetine, cells were electroporated with 1mg/mL OVA and introduced into recipient mice that had previously received CFDA-SE-labeled transgenic CD8+ T cells from OT-I mice specific for $OVA_{257\text{-}264}$-K^b complexes. Under the conditions used, β_2-microglobulin negative cells that were incubated with OVA but not electroporated failed to induce proliferation of OT-I.SJL cells following immunization. In addition, β_2-microglobulin negative cells electroporated with concentrations of OVA lower than

Figure 3.14

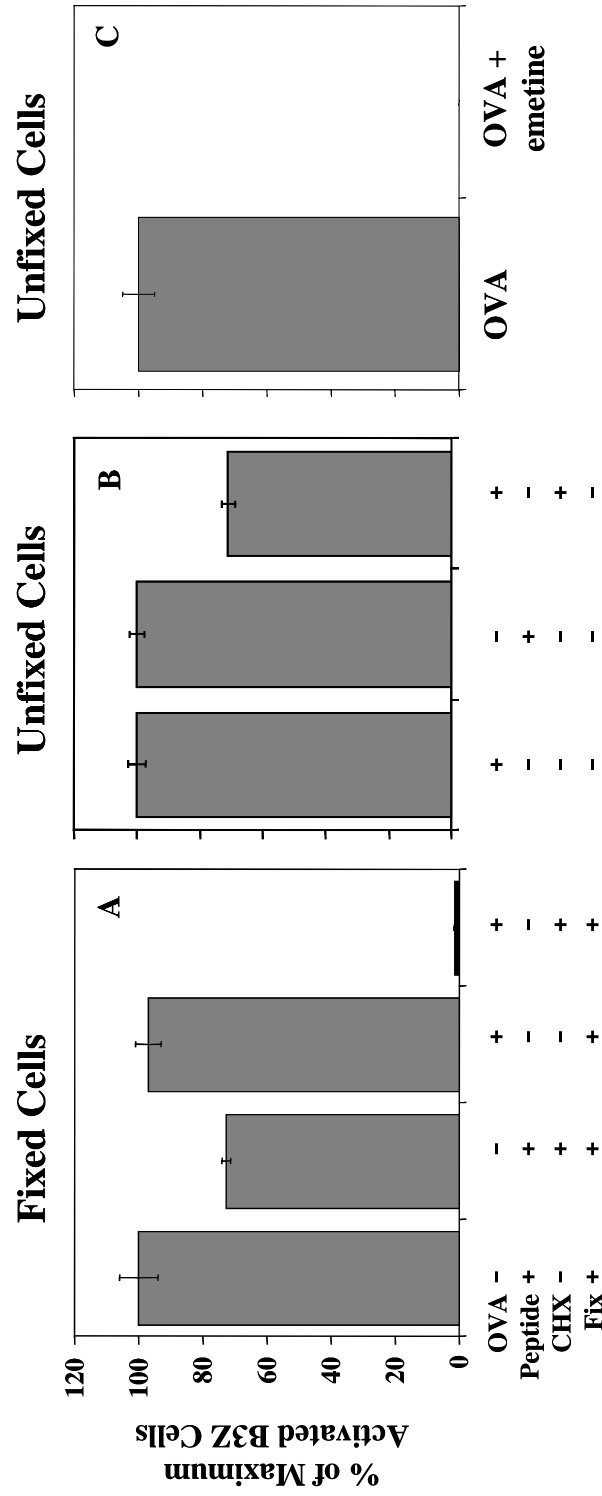

Figure 3.14 Direct presentation requires cellular protein synthesis.

WT3 cells were treated with cycloheximide for 1 hr prior to electroporation with 10mg/mL OVA. Cells were then treated for an additional 5 hr with cycloheximide (A, B) or with emetine (C) before being washed (B, C) or fixed (A). Peptide pulsed cells were pulsed with 1ng/mL $OVA_{257-264}$ 30 minutes prior to being incubated with B3Z cells. Cells were incubated overnight with B3Z cells then stained with X gal. Each panel is representative of 4 experiments. Fixed and unfixed cells are presented on different panels because the stimulator to responder ratio was different. The same number of stimulator cells was initially plated, but a greater number of cells were lost in the fixed wells than were lost in the unfixed wells due to the additional washes. Error bars represent the standard error of the mean.

1mg/mL did not reproducibly induce proliferation of OT-I.SJL cells, indicating that under these conditions antigen was limiting (Donohue et al., 2006). Seventy-two hours after immunization with β_2-microglobulin negative cells, OT-I.SJL activation was measured by dilution of CFDA-SE fluorescence due to proliferation (Figure 3.15B-D). Comparable OT-I.SJL proliferation was seen after immunization with β_2-microglobulin negative cells that were treated or untreated with emetine. Thus, protein synthesis is not required in the antigen donor cell for cross-priming to occur *in vivo*.

3.2.3.4 Requirement for Vesicular Transport in the Antigen Donor Cell

The majority of our *in vivo* studies have utilized OVA, a secreted protein (Braell and Lodish, 1982; Meek et al., 1982; Palmiter et al., 1978), as our model antigen. It is conceivable that as a secreted protein, OVA might make a better substrate for cross-priming than cellular proteins. The experiments using IAV nuclear protein (Figure 3.8) or β gal (Figures 3.13 and 3.15) as the model antigen, as well as studies using LCMV as the model antigen (Pozzi et al., 2005), suggest that non-secreted proteins can serve as substrates for the cross-priming pathway. However, the ability of IAV nuclear protein and β gal to serve as substrates for the cross-priming pathway does not preclude a role for secretion or the secretory pathway in the transfer of antigen from the donor cell to the presenting cell. Thus, we sought to assess the role of vesicular transport in the donation of antigen during cross-priming by treating β_2-microglobulin negative cells with Brefeldin A (BFA) to prevent vesicular transport. BFA prevents vesicular transport by preventing the formation of non-clathrin coated vesicles in the ER by one of two mechanisms. One, by binding to the nucleotide exchange factor on ARF-1 (Donaldson et al., 1992), which is the first step of recruiting coatomer (non-clathrin proteins required for budding) subunits to the

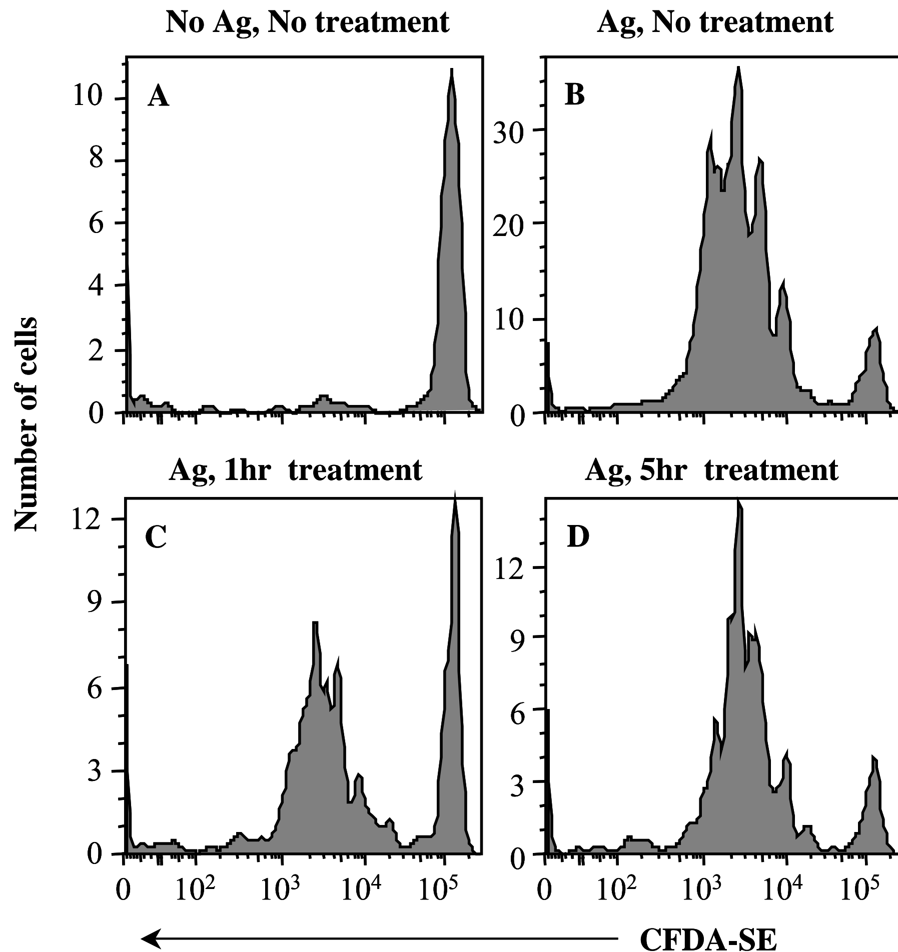

Figure 3.15 Cross-priming in the absence of protein synthesis.

Mice that had received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells were immunized with β_2m_{neg} cells that were mock electroporated (A) or electroporated in the presence of 1mgs/mL OVA (B-D). The cells were either untreated (B) or treated with the protein synthesis inhibitor emetine for 1 hr (C) or 5 hr (D) prior to electroporation. Each panel is representative of 4 experiments.

Golgi. Alternatively, BFA prevents vesicular transport by binding βCOP, a component of coatomer, and preventing coatomer assembly (Donaldson et al., 1990; Donaldson et al., 1991). Because BFA is readily reversible, the antigen donor cells needed to be fixed to prevent restoration of vesicular transport once they were injected into mice. As mentioned above, paraformaldehyde fixation can also be used to prevent vaccinia virus replication (Figure 3.1) (Hulskotte et al., 1997). Thus it was necessary to demonstrate that fixed cells could donate antigen for cross-priming. β_2-microglobulin negative cells were infected with an rVV construct expressing full length OVA (see Figure 3.2) and then treated with either psoralen UV-irradiation or 2% paraformaldehyde. Infected β_2-microglobulin negative cells were then used to immunize mice that had previously received an adoptive transfer of CFDA-SE-labeled transgenic CD8+ T cells from OT-I.SJL mice specific for $OVA_{257-264}$-K^b complexes. Seventy-two hours after immunization with the β_2-microglobulin negative cells, OT-1.SJL proliferation was measured by dilution of CFDA-SE label due to proliferation. While psoralen UV-irradiated cells were able to induce a higher level of proliferation than the 2% paraformaldehyde fixed cells, sufficient proliferation was observed in response to the fixed cells to indicate that they were able to induce cross-priming *in vivo* (Figure 3.16).

Several reports have indicated that the secretory rearrangements caused by BFA can inhibit viral replication (Cuconati et al., 1998; Doedens et al., 1994; Gazina et al., 2002; Jensen and Norrild, 2002) and protein synthesis, likely because of the induction of ER stress responses (Fishman and Curran, 1992; Mellor et al., 1994). Thus, we tested the effects of BFA treatment on rVV infection and protein expression. β_2-microglobulin negative cells were infected with an rVV expressing a chimeric fluorescent protein (NP-S-GFP) (Figure 3.2) for 1 hour and then either untreated or treated with 5μg/mL BFA for an additional 4 hours. Protein expression was determined by the percent of fluorescent cells while protein expression was determined by the

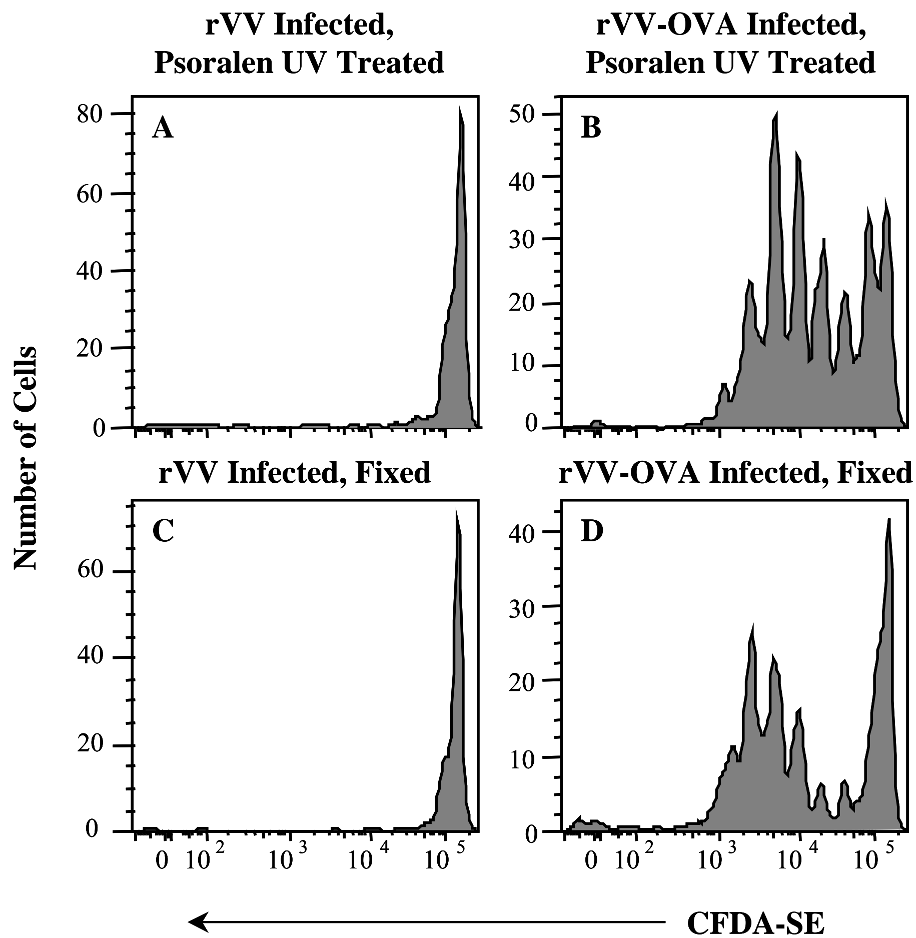

Figure 3.16 Ability of paraformaldehyde fixed cells to act as antigen donor cells *in vivo*.

B6 mice that had received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells were immunized with rVV (A, C) or rVV-OVA (B, D) infected β_2m_{neg} cells that were either treated with psoralen UV-irradiation (A, B) or with 2% paraformaldehyde fixation (C, D). Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Panel is representative of 4 experiments.

mean fluorescence of the cells, which is used as a measure of chimeric protein produced. Comparable levels of infection and protein expression were observed in the untreated and BFA treated cells (Figure 3.17), indicating that BFA does not appreciably affect the ability of the rVV to infect or express protein within these cells.

To assess the requirement for vesicular transport in the donation of antigen in cross-priming, rVV infected β_2-microglobulin negative cells were either untreated or treated with 5μg/mL BFA for 4 hours after infection and then fixed with 2% paraformaldehyde. Fixed cells were then injected IV into C57BL/6 mice that had previously received an adoptive transfer of CFDA-SE labeled OT-I.SJL cells. Seventy two hours later, OT-I.SJL activation was measured by loss of the CFDA-SE signal due to proliferation. Comparable levels of proliferation were observed whether or not the infected β_2-microglobulin negative cells were treated with BFA (Figure 3.18). To control for the efficacy of the BFA used, an *ex vivo* ICS performed on the same day as the β_2-microglobulin negative cells were treated was included (Figure 3.18E). ICS depends on the sequestering of cytokine within the cell, thus a successful ICS indicates that the BFA used was efficacious. This result indicates that vesicular transport in the antigen donor cell is not required for cross-priming *in vivo*.

3.3 Discussion

Rational vaccine design requires a mechanistic understanding of the antigen processing and presentation pathways available to prime T cells. In this study, we set out to better characterize the requirements of the cross-priming pathway and to contrast these requirements to those of the direct priming pathway. The first element of this study examined the form of antigen transferred to the antigen presenting cell in cross-priming *in vivo*. As mentioned in the introduction, the form of antigen transferred *in vivo* is the source of controversy, with initial studies suggesting

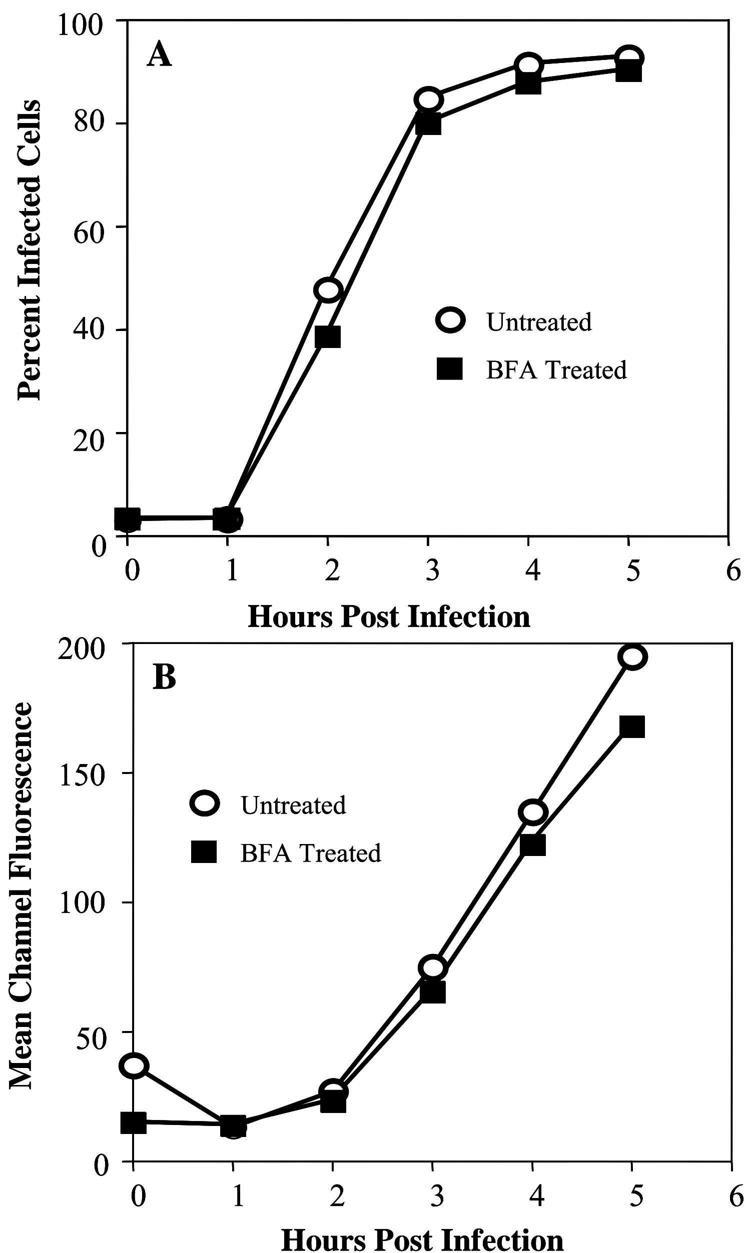

Figure 3.17 Determining the effects on viral infection of and protein expression in cells treated with BFA.

β_2m_{neg} cells were infected with an rVV expressing a chimeric fluorescent protein, NP-S-GFP. One hour after infection, infected cells were either untreated (open circles) or treated (black squares) with 5μg/mL BFA. Protein expression was monitored by GFP fluorescence and was determined by flow cytometry. (A) Infection was measured by the percent of fluorescent cells while (B) protein expression was measured by mean channel fluorescence. Each panel is representative of two experiments.

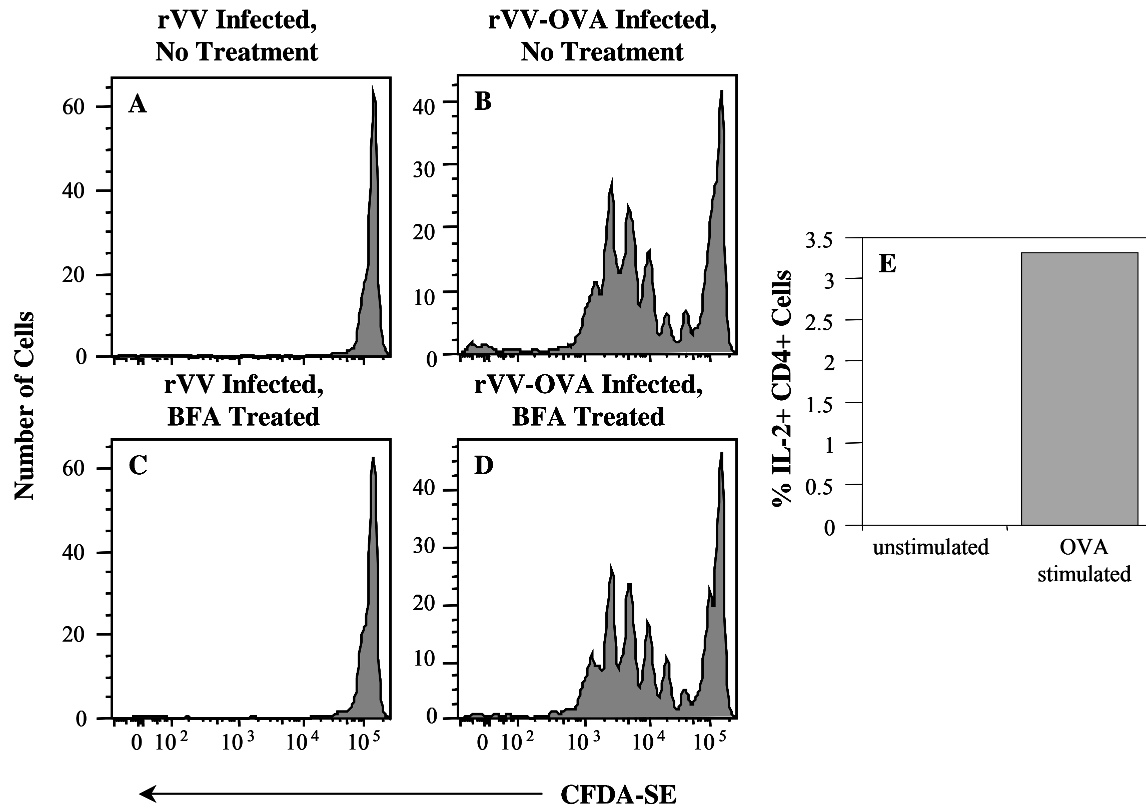

Figure 3.18 Cross-priming in the absence of vesicular transport.

B6 mice that had received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells were immunized with rVV (A, C) or rVV-OVA (B, D) infected β_2m_{neg} cells either untreated (A, B) or treated (C, D) with BFA and fixed in paraformaldehyde. Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Panel is representative of 4 experiments. (E) A panel from an *ex vivo* IL-2 ICS performed on the same day as the β_2m^{neg} cells were treated with BFA is included as a control to demonstrate the efficacy of the BFA. Similar controls were run with all 4 BFA experiments.

particulate or aggregated protein (Falo et al., 1995; Kovacsovics-Bankowski et al., 1993; Rock and Clark, 1996; Shi and Rock, 2002) and more recent studies suggesting chaperone-bound peptides as the form of antigen transferred *in vivo* (Arnold et al., 1995; Binder and Srivastava, 2005; Suto and Srivastava, 1995; Udono and Srivastava, 1993). Thus, we examined whether protein degradation intermediates or minimal antigenic peptides could serve as the form of antigen transferred *in vivo*. As shown in Figure 3.6, there is an inverse relationship between the stability of the antigen and the ability to induce cross-priming. Despite the fact that antigenic peptides are generated from the rapidly degraded proteins at three times the rate at which they are generated from the stable construct (Princiotta et al., 2003), no cross-priming was observed from cells expressing the rapidly degraded protein. Cross-priming could, however, be observed from the rapidly degraded protein if proteasomal degradation was inhibited in the antigen donor cell (Figure 3.6B). Similarly, minimal antigenic peptides were also unable to stimulate cross-priming *in vivo* (Figures 3.3 and 3.4). A previous study using peptide pulsed splenocytes could not detect transfer of peptide to host antigen presenting cells *in vivo* (Livingstone and Kuhn, 2002). This inability to detect the transfer of peptides in this study may have been due to the extremely short half-life of peptides in the absence of MHC class I molecules that bind to and protect the peptide from degradation (Reits et al., 2003; Saric et al., 2001). However, MHC class I-mismatched splenocytes pulsed with peptide failed to stimulate cross-priming *in vivo* at any peptide concentration (Figure 3.3) in this study; demonstrating that cells pulsed with exogenous peptide are incapable of stimulating cross-priming *in vivo*. Contrary to the chaperone-bound peptide hypothesis, neither cytosolic nor ER-targeted minimal antigenic peptides expressed within the donor cell (Figure 3.4) were capable of stimulating cross-priming despite the fact that under both conditions, these peptides had access to the ER and the molecular chaperones hypothesized to be required for cross-priming (Day et al., 1997). These findings agree with those of other groups (Shen and Rock, 2004; Wolkers et al., 2004) and extend the findings of a prior study that

demonstrated that cross-priming does not require the transport of peptides into the ER by TAP (Schoenberger et al., 1998) in antigen donor cells. These findings led us to examine whether other antigen processing and presentation functions are required within the antigen donor cell.

The first such process examined was the requirement for proteasomal degradation with the antigen donor cell. Figure 3.8 demonstrates that proteasomal degradation is not required in the antigen donor cell and that inhibition of the proteasome may even enhance cross-priming (Figure 3.8A and 3.8C). This observation further strengthens the findings described above and implies that stable protein, rather than peptides, is the form of antigen transferred from the antigen donor cell to the antigen presenting cell in cross-priming *in vivo*.

A previous study demonstrated that neosynthesis of antigen was required for direct priming (Khan et al., 2001), a pathway that is enhanced by the rapid degradation of antigen. Thus, the observation that stable protein, rather than peptides or degradation intermediates, is the form of antigen transferred in cross-priming led us to examine whether neosynthesis of antigen was required for donation to the cross-priming pathway. To examine this requirement, we created a cell line, L4.2, which expressed an enzymatically active antigen, β gal, under an inducible promoter to permit us to examine the requirement for new synthesis of antigen in both direct and cross-priming. Using L4.2 cells as the antigen donor cells, we demonstrated that *in vivo* cross-priming does not require new synthesis of antigen (Figure 3.13). This was in direct contrast to direct-presentation, which was greatly enhanced by new synthesis of antigen and almost undetectable in its absence (Figure 3.12). This latter finding concurs with the findings of Khan et al, although our system allows for more sensitive detection of antigen than that used by Khan *et al.* (Khan et al., 2001). Our observation that newly synthesized antigen greatly enhances direct presentation further implicates Defective Ribosomal Products (DRiPs) as the predominant source of antigenic peptides for direct priming (Reits et al., 2000; Schubert et al., 2000). DRiPs are generated from newly synthesized polypeptides that are unable to reach their native state due to

mistranslations, truncations, improper folding, or improper post-translational modifications. DRiPs have an extremely short half-life and can only be visualized by inhibiting their degradation (Schubert et al., 2000). Therefore our measurements of enzymatically active β gal are unlikely to include DRiPs. However, it is clear from our data that under conditions at which direct presentation is barely detectable (Figure 3.12), antigen is available and sufficient for donation during *in vivo* cross-priming (Figure 3.13). This implies that cross-priming can utilize pools of antigen that are not available for the direct presentation pathway.

The observation that new synthesis of antigen is not required in the antigen donor cell *in vivo*, further discounts the chaperone-bound peptide hypothesis of cross-priming. Our data reveal that 16 hours after the cessation of the production of enzymatically active β gal, antigen is still available for donation during cross-priming (Figures 3.10B and 3.13). Even allowing for the fact that production of DRiPs, which are undetectable in our system, may proceed for a period of time after the production of intact protein, peptide stabilized by chaperones would be required to linger within the cells for many hours prior to antigen donation. Chaperones are known to transiently associate with unfolded proteins to either target their destruction (Kleizen and Braakman, 2004; Molinari et al., 2002) or to assist their acquisition of a functional conformation (Martin et al., 1991; Ostermann et al., 1989). While chaperones are known to associate constitutively with some substrates, such as the glucocorticoid receptor (Dittmar et al., 1997), these associations appear to be highly specialized interactions that are an exception rather than a rule. Thus, the requirement that a varied assortment of antigens be available during cross-priming would necessitate chaperone-antigen interactions that were both prolonged and promiscuous on the part of the chaperone. Such interactions would be costly to the cell as they would sequester chaperones from their normal housekeeping functions. Thus, it is extremely unlikely that chaperone-bound peptides produced within our system would linger for the 16 plus hours

necessary for cross-priming to occur; further implicating stable proteins as the source of transferred antigen in cross-priming *in vivo*.

Both donor cell-encoded proteins (Albert et al., 1998a) and the phenotypic status of the donor cell (Albert et al., 1998b; Bellone et al., 1997; Gallucci et al., 1999) have both been implicated in the donation of antigen to antigen presenting cells. Cells may synthesize proteins that modulate cross-priming by increasing the uptake of antigen, either at the level of cellular clearance or antigen transfer to APC, or by modulating pAPC phenotype to enhance antigen presentation. Using a system in which the antigen is electroporated into the APC (direct presentation) or the antigen donor cell (cross-priming), we were able to examine the requirement for protein synthesis in the absence of the requirement for synthesis of the antigen. In contrast to direct presentation, which was completely abrogated by the inhibition of protein synthesis (Figure 3.14), inhibition of protein synthesis in the antigen donor cell had no effect on cross-priming (Figure 3.15). Having previously demonstrated that neosynthesis of antigen greatly enhances direct presentation, it would seem obvious that no direct presentation would be observed in the absence of protein synthesis. However, as noted in the results section, we increased the concentration of ovalbumin used in these direct presentation studies to take advantage of the small amount of direct presentation that occurs in the absence of new synthesis of antigen. The lack of direct presentation when protein synthesis was blocked likely represents a requirement for synthesis of components of the MHC class I processing pathway. The requirement for protein synthesis in direct presentation is further emphasized by the observation that shut down of host protein synthesis is a primary mechanism of immune evasion utilized in viral infection. Yet it is clear that antigen donation does not have a requirement for ongoing protein synthesis, permitting antigen presentation even under conditions of viral immune evasion. Furthermore, antigen donation occurred even when antigen was added to donor cells 5 hours after protein synthesis was blocked (Figure 3.15D), indicating that cellular proteins with a half-life of less than 5 hours are

likely not involved in antigen donation; once again implicating stable protein as the form of antigen transferred during cross-priming *in vivo*. This finding also suggests that cross-priming may be a passive process for the antigen donor cell. In the context of a viral infection, this means that presentation of viral antigens can occur even when the infected cell lacks protein synthesis.

We next examined the requirement for secretory function for the donation of antigen from the antigen donor cell for *in vivo* cross-priming. The requirement for secretory function was initially of interest because the majority of our cross-priming studies utilized ovalbumin, a secreted protein (Braell and Lodish, 1982; Meek et al., 1982; Palmiter et al., 1978). Yet a cytosolic protein, β gal, and a nuclear protein, IAV NP, were also able to mediate cross-priming *in vivo* (Figures 3.8 and 3.13). Still, with the mechanism of antigen transfer unknown, it was conceivable that vesicular transport was required in the antigen donor cell in order for antigen donation. Vesicular transport could be involved in secreting the antigen transferred or it could be involved in cell surface transport of other molecules which may be necessary for the transfer of antigen to the antigen presenting cell. Surprisingly, antigen donation was not inhibited when the secretory pathway was inhibited using Brefeldin A treatment (Figure 3.18), demonstrating that vesicular transport is not required in the antigen donor cell for antigen donation to occur. This finding further emphasizes that cross-priming may be passive process for the antigen donor cell.

In summary, we've demonstrated that the form of antigen transferred during *in vivo* cross-priming is long-lived stable protein. This is in contradiction to the chaperone-bound peptide hypothesis. Our findings do not eliminate the possible participation of molecular chaperones in cross-priming *in vivo*. However, chaperone-bound peptides would have to linger within the antigen donor cell long enough to be transferred, a time course which seems unlikely due to the transient binding of peptides by chaperones and the short half-life of peptides within the cell. Although we have demonstrated that chaperone-bound peptides are not the form of antigen transferred in cross-priming *in vivo*, chaperone-bound peptides may still have a

therapeutic effect in stimulating T cell responses to peptides derived as tumors. As such, the use of chaperone-bound peptides should continue to be explored. Rational vaccine design, however, should take protein stability into account when targeting proteins to the direct- or cross-priming pathways. Direct-priming uses newly synthesized Defective Ribosomal Products and requires ongoing protein synthesis of factors other than the antigen for T cell activation. Cross-priming, however, utilizes long-lived stable protein and has no requirement for proteasomal processing, protein synthesis or vesicular transport of the antigen or other cellular factors in the antigen donor cell. These findings suggest that the cross-priming pathway evolved to allow the generation of immune responses to pathogens that were not directly presented either due to the inability to infect antigen presenting cells or due to expression of immune evasion molecules that subvert the direct priming pathway. The lack of requirement for any known cellular processes to transfer antigen makes the cross-priming pathway resistant to modification and manipulation. By exploiting the differences in these two pathways, rational vaccine design has the potential to design vaccines that take advantage of both pathways, increasing the likelihood of a successful vaccine to stimulate CD8+ T cells.

Chapter 4

Vaccinia Virus Expression Permits Soluble Antigen to Access the TAP-Independent Cross-Priming Pathway

4.1 Introduction

Recombinant viral vectors expressing transgenes from other diseases have been proposed as a means of stimulating CD8+ T cell immunity to the transgene *in vivo*. While several studies have demonstrated that recombinant viral vectors can effectively express and stimulate immunity to a transgene (Cooney et al., 1991; Kahn et al., 2001; Konishi et al., 1998; Ockenhouse et al., 1998; Ramsburg et al., 2004; Reuter et al., 2002; Roberts et al., 1998; Roberts et al., 2004; Rose et al., 2001; Schlereth et al., 2000), a systematic study comparing the antigen processing pathways available to a transgene when expressed by different viral vectors has not been done. Our goal is to examine the MHC class I antigen processing pathways available to a model antigen when expressed by either recombinant vaccinia virus (rVV) or recombinant vesicular stomatitis virus (rVSV), two of the recombinant viral vectors often proposed as vaccine vectors.

Many studies have utilized rVV or rVSV as vaccine vectors to stimulate CD8+ T cell immunity to a transgene (Cooney et al., 1991; Haglund et al., 2002; Konishi et al., 1998; Ockenhouse et al., 1998; Rose et al., 2001). The advantage to using these viruses is that they are both able to accept large transgenes, at least 25kb for rVV (Perkus et al., 1991; Perkus et al., 1985) and 2.5kb for rVSV (Schnell et al., 1996); and are able to be grown to high titer. In this study, we will use rVV and rVSV to express the model antigen ovalbumin (OVA). Unlike many viral proteins used as model antigens, OVA is a relatively inert protein; expression of OVA is not expected to exert an effect on the virus used to express it. In addition, the CD8+ T cell response

to OVA has been extensively characterized (Raychaudhuri et al., 1992; Staerz et al., 1987) and we can use transgenic mice that express a transgenic T cell receptor specific for $OVA_{257-264}$ complexed to K^b (Clarke et al., 2000).

Both VV and VSV stimulate virus-specific CD8+ T cell responses, although the absolute requirement for the CD8+ T cell response in controlling either VV or VSV infection remains controversial. VV-specific CD8+ T cells are readily detected after VV infection and persist for decades (Demkowicz et al., 1996; Hammarlund et al., 2003; Littaua et al., 1992). While VSV-specific CD8+ T cells are also readily detected post-infection (Hale et al., 1978; Lefrancois and Lyles, 1983; Puddington et al., 1986), no studies have investigated how long post-infection VSV-specific CD8+ T cells linger. CD8+ T cells do not appear to be essential for controlling either VV or VSV infection; mice lacking CD8+ T cells, but otherwise immunocompetent, were able to survive lethal challenge (Spriggs et al., 1992b; Thomsen et al., 1997; Xu et al., 2004). However, in the absence of CD4+ T cells or humoral immunity, CD8+ T cells were sufficient to protect against a lethal VV or VSV challenge (Andersen et al., 1999; Thomsen et al., 1997; Xu et al., 2004), implying that while a CD8+ T cell response to these viruses may be superfluous, it is nevertheless beneficial. Memory VSV-specific CD8+ T cells may also play a role in preventing infection by other VSV strains, a function that cannot be mediated by VSV strain-specific humoral immunity (Burkhart et al., 1994b; Hale et al., 1981; Kundig et al., 1993a; Lefrancois and Lyles, 1983).

MHC class I processing begins with the proteolytic degradation of protein antigens by the proteasome (Dick et al., 1996; Niedermann et al., 1995; Paz et al., 1999; Qian et al., 2006) or other proteases (leucine amino-peptidase, furin, thimet oligopeptidase, and TPPII) (Beninga et al., 1998; Geier et al., 1999; Gil-Torregrosa et al., 1998; Silva et al., 1999) in the cytosol. Degradation products are then transported into the ER via TAP (Androlewicz et al., 1993; Heemels et al., 1993; Neefjes et al., 1993) or via the translocon (Henderson et al., 1992; Meacock

et al., 2000; Smith et al., 2002a; Wei and Cresswell, 1992; Wolfel et al., 2000). In the ER, MHC class I and TAP molecules form a complex in the presence of tapasin and other chaperones. Following binding of peptides, stable MHC class I-peptide complexes are exported to the plasma membrane via the constitutive secretory pathway (Neefjes and Ploegh, 1988; Ortmann et al., 1994; Suh et al., 1994; Wearsch and Cresswell, 2007). Presentation of OVA has been demonstrated to be TAP-dependent *in vitro* (Norbury et al., 2001). However, Norbury *et al.* also demonstrated that the presentation of OVA expressed from rVV was TAP-independent *in vivo* (Norbury et al., 2001). We hypothesize that viral expression of OVA will affect the antigen processing and presentation pathways available to OVA. Thus, in this study we will examine the antigen processing and presentation pathways available to OVA expressed by rVV or rVSV by comparing the ability of rVV or rVSV expressed OVA to stimulate OVA-specific transgenic T cells by both direct and cross-priming in both wild-type and TAP knock-out mice.

4.2 Results

4.2.1 Titrating Viral Dose *In Vivo*

In order to investigate and compare the ability of rVV or rVSV expressed OVA to stimulate T cells *in vivo*, we needed to determine the optimal dose. For our experiments, the optimal dose is defined as the viral dose at which the CD8+ T cell response to the transgene is maximized compared to the CD8+ T cell response to the viral vector. Thus, B6 (wild type) mice were infected intravenously (IV) with rVV-OVA or rVSV-OVA viral doses ranging from 2.5×10^3 PFU/mouse through 2.5×10^7 PFU/mouse. IV immunization results in a splenic presentation of antigen, allowing splenic CD8+ T cells responses to be examined (Aichele et al., 2003; Weiss, 1990). Six days later, virus-specific and OVA-specific splenic CD8+ T cell responses were

measured by intracellular cytokine staining (ICS) for interferon-γ (IFN-γ). Both the percent of CD8+ T cells and the number of CD8+ T cells producing IFN-γ were determined in response to the transgene or to the viral vector. As seen in Figure 4.1, both the maximum percent (Figure 4.1A) and number (Figure 4.1B) of VV-specific and OVA-specific cells was seen at a viral dose of 2.5×10^6 PFU for VV-OVA. Figures 4.1C and 4.1D show that the maximum percent (Figure 4.1C) and number (Figure 4.1D) of VSV-specific and OVA-specific cells were seen at a viral dose of 2.5×10^5 PFU for VSV-OVA. Thus, 2.5×10^6 PFU/mouse and 2.5×10^5 PFU/mouse were chosen as the optimal dose for rVV-OVA and rVSV-OVA respectively. These optimal doses were used in all subsequent experiments in wild-type mice.

4.2.2 CD8+ T Cell Response in Wild Type Mice

Once the optimal dose for rVV-OVA and rVSV-OVA was determined, we sought to compare the naïve immune response to OVA in wild type mice. Wild type mice were infected IV with the optimal dose of rVV-OVA or rVSV-OVA. The parent vectors lacking the OVA transgene, subsequently referred to as rVV and rVSV, were also included as negative controls for the OVA response (Figure 4.2). Six days later, virus-specific and OVA-specific CD8+ T cell responses were measured by ICS for IFN-γ. Both the percent of IFN-γ+ CD8+ T cells and the number of IFN-γ+ CD8+ T cells were determined. rVSV-OVA infection stimulated 2.8% of CD8+ T cells to produce IFN-γ, compared to the less than 0.5% stimulated by rVV-OVA (Figure 4.2A), a nearly 6-fold increase. The number of CD8+ T cells stimulated to produce IFN-γ by rVSV-OVA was similarly increased, with 5-fold more cells being stimulated by rVSV-OVA than were stimulated by rVV-OVA (Figure 4.2B).

Only a single peptide from each parent virus was used to evaluate the virus-specific

Figure 4.1

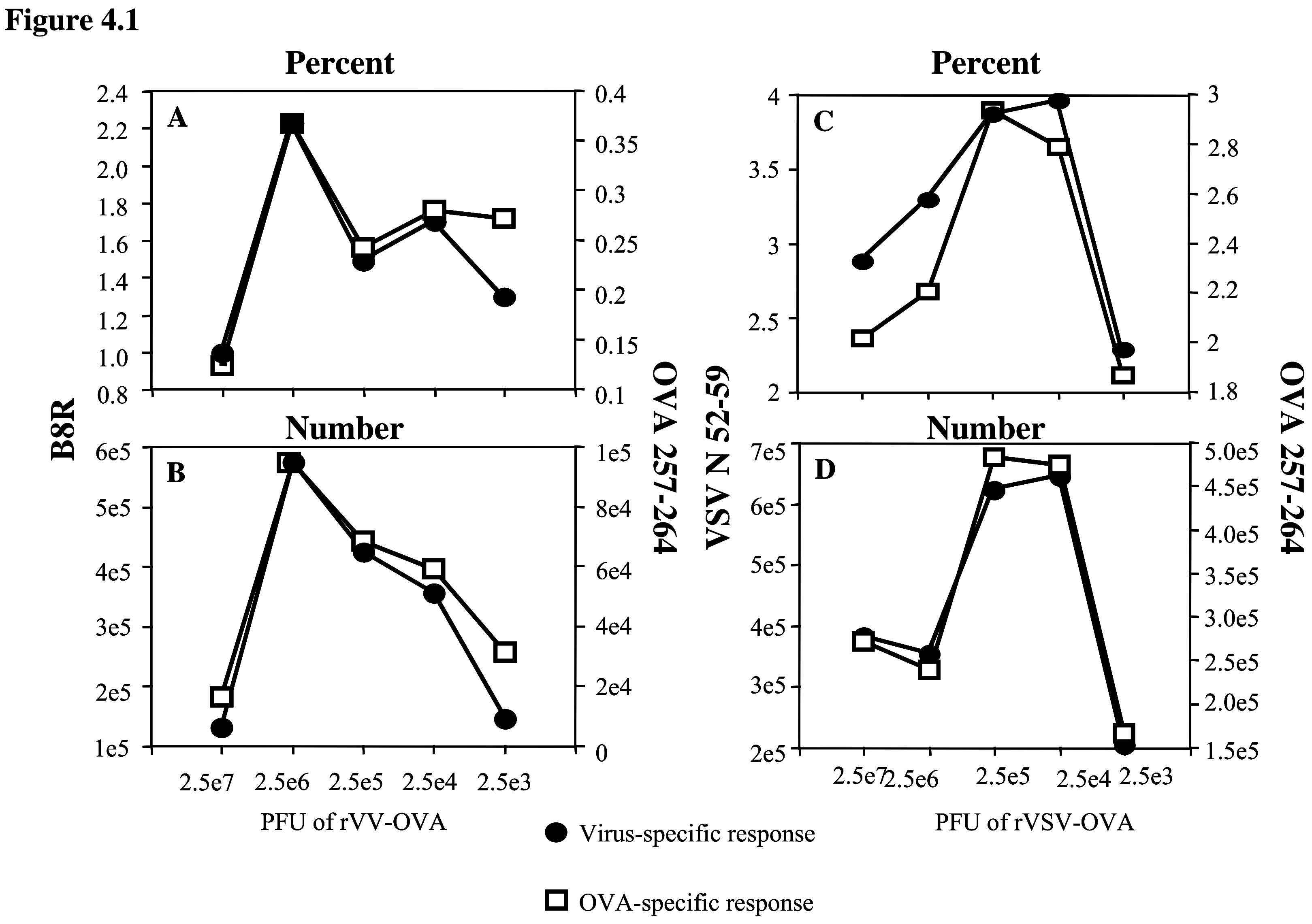

Figure 4.1 Titration of viruses to maximize the response to OVA.

Mice were immunized with the indicated dose of rVV-OVA (A, B) or rVSV-OVA (C,D) . Six days later, the percent (A, C) or number (B, D) of responding CD8+ T cells present in spleens were determined by their expression of IFN-γ in response to cells pulsed with either a virus specific peptide (B8R for rVV and VSV N_{52-59} for rVSV) or pulsed with $OVA_{257-264}$. Closed circles indicate response to virus specific peptide pulsed cells while open squares indicate response to $OVA_{257-264}$ pulsed cells. This experiment was repeated twice with similar results.

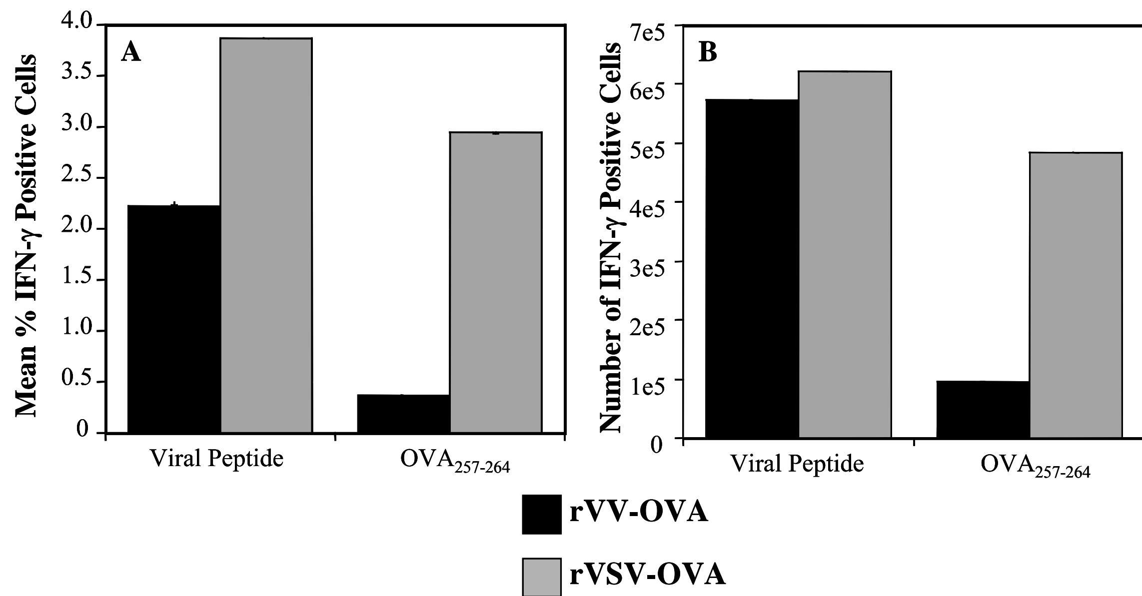

Figure 4.2 Immune response to optimal titers of virus in naïve mice.

(A and B) B6 mice were immunized with either 2.5×10^6PFU rVV-OVA or 2.5×10^5PFU rVSV-OVA. Six days later, the percent (A) or number (B) of responding CD8+ T cells present in spleens were determined by their expression of IFN-γ in response to cells pulsed with either a virus specific peptide (B8R for rVV and VSV N_{52-59} for rVSV) or pulsed with $OVA_{257-264}$. T cells isolated from rVV-OVA infected mice are indicated by black bars while those isolated from rVSV-OVA infected mice are indicated by gray bars. Results are representative of 3 experiments; errors bars show ±SEM.

CD8+ T cell response, so the results from rVV cannot be directly compared with those from rVSV as they only reflect a fraction of the total viral immune response. However, the similar numbers of virus-specific CD8+ T cells stimulated by both rVV-OVA and rVSV-OVA (Figure 4.2B) indicates that the lesser OVA-specific response seen in rVV-OVA is not due to lack of infection. This implies that OVA expressed by rVSV is better at stimulating OVA-specific CD8+ T cells than OVA expressed by rVV.

4.2.3 Expression of OVA by rVV and rVSV

To determine whether the mechanism behind the greater number of CD8+ T cells stimulated by rVSV-OVA was due to differing levels of OVA expression, we performed a Western blot on rVV-OVA and rVSV-OVA infected fibroblasts. rVV and rVSV infected fibroblasts were included as negative controls while 50ng of OVA was included as a positive control. Two separate infection time points were used to optimize OVA expression from each of the viruses. Based on initial experimental results using rVV-OVA infected fibroblasts, it was necessary to dilute the rVV-OVA infected cells in order to measure the amount of OVA produced. These gels indicate that rVV-OVA infected cells express more OVA than rVSV-OVA infected cells, especially considering that rVV-OVA infected cells were diluted one thousand-fold prior to loading (Figure 4.3). Thus, OVA expression is at least 1000-fold higher in cells infected with rVV-OVA than those infected with rVSV-OVA. These results indicate that the enhanced OVA-specific CD8+ T cell response stimulated by rVSV-OVA infection (Figure 4.2) could be due to the lower level of OVA expression by rVSV-OVA. Studies have demonstrated that mature CD8+ T cells can accommodate an activation signal of restricted intensity; surpassing the activation signal intensity, however, results in deletion of the cell (Alexander-Miller et al., 1996; Kroger and Alexander-Miller, 2007; Rocha et al., 1995). Therefore, the high concentration of

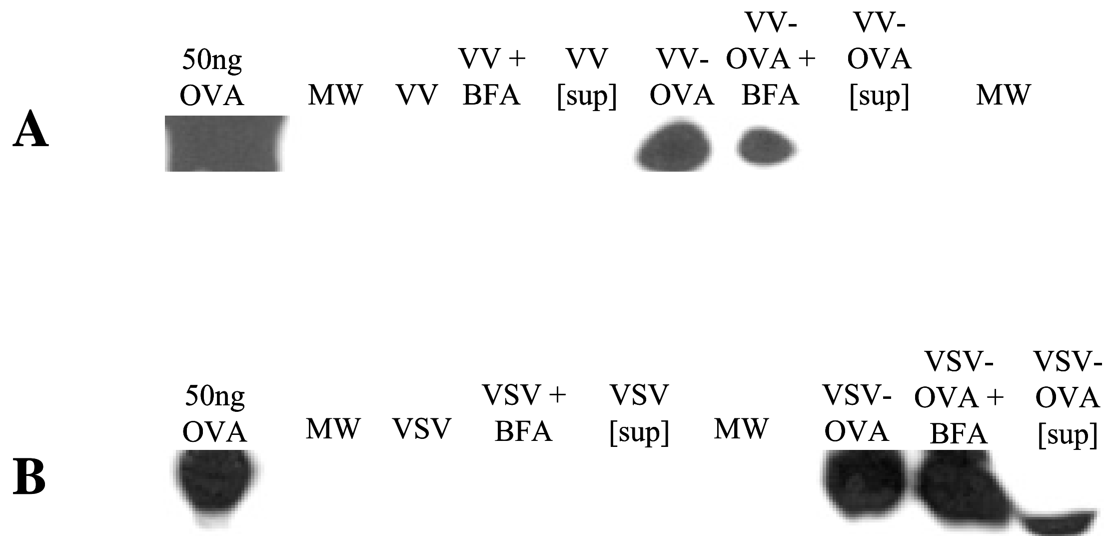

Figure 4.3 Western blots showing OVA expression by rVV-OVA and rVSV-OVA.

$1x10^5$ fibroblasts were infected with an MOI=10 with rVV or rVV-OVA for 4 hours (A) or with rVSV or rVSV-OVA for 16 hours (B). Cells were spun down after infection and either diluted 1:1000 in lysis buffer (A) or resuspended directly in lysis buffer (B) and loaded onto the gel as indicated. +BFA indicates that cells were treated with 5µg/mL Brefeldin A 1 hour post-infection (A) or 4 hours post-infection (B) while [sup] indicates supernatant from infected cells that was concentrated in order to concentrate any OVA secreted from the infected cells into the media. Gels shown are representative of 3 separate experiments.

OVA expressed by rVV-OVA could result in a reduced number of CD8+ T cells specific for rVV-expressed OVA. In addition to measuring the amount of OVA expressed from rVV-OVA and rVSV-OVA, the secretion of OVA was also determined. While OVA is a secreted protein (Braell and Lodish, 1982; Meek et al., 1982; Palmiter et al., 1978), the secretion of OVA may be altered by the viral infection, resulting in the different level of response we observed in naïve wild type mice (Figure 4.2). To determine whether OVA was secreted from infected cells, we either sequestered OVA within the infected cells by addition of Brefeldin A (BFA) or concentrated the OVA secreted into the media of infected cells by loading supernatant harvested from infected cells onto a Centricon Centrifugation device. BFA treatment reduced the amount of OVA present in VV-OVA infected cells. This reduction is likely due to BFA treatment inhibiting protein synthesis (Fishman and Curran, 1992; Mellor et al., 1994). However, no OVA was seen in the supernatant of rVV-OVA infected cells, indicating that the OVA produced by rVV-OVA infection is not secreted, but is sequestered within the infected cell (Figure 4.3A). In contrast, rVSV-OVA infected fibroblasts were able to secrete OVA, as is indicated by the increase in the OVA band in the BFA treated cell lane as well as by the OVA band seen in the concentrated supernatant (Figure 4.3B). These results indicate that the enhanced OVA-specific CD8+ response stimulated by rVSV-OVA infection (Figure 4.2) could also be due to the secretion status of OVA.

4.2.4 MHC Class I Processing Pathways Available *In Vivo* to OVA Expressed by Different Viruses

Examination of the MHC class I processing pathways available to virally expressed OVA requires us to examine the CD8+ T cell response in relation to antigen presentation. Thus, it is necessary to use T cells from transgenic mice, OT-I mice (Clarke et al., 2000), that express a

transgenic T cell receptor specific for $OVA_{257-264}$ complexed to K^b. Adoptively transferring transgenic T cells specific for OVA results in a high frequency of T cells specific for OVA (Pape et al., 1997), permitting us to examine T responses in relation to antigen presentation without having to wait for several rounds of division and acquisition of effector function. Additionally, by using OT-I mice that had been crossed with B6.SJL mice (OT-I.SJL), we could distinguish the adoptively transferred cells by staining for the CD45.1 marker, which is expressed by the adoptively transferred cells but not by the recipient B6 mice. Before we examine the processing pathways available to virally expressed OVA, it was first necessary to examine the response of the OT-I.SJL T cells in wild type mice which have all the MHC class I processing pathways available. Thus, we adoptively transferred lymphocytes harvested from OT-I.SJL mice into wild-type mice. Mice that received adoptive transfers were subsequently infected with the optimal dose of rVV-OVA or rVSV-OVA. rVV and rVSV infected mice were included as negative controls. Proliferation of the adoptively transferred OT-I.SJL cells was then used as a measure of CD8+ T cell activation (Figure 4.4). OT-I.SJL cells proliferated similarly in response to OVA expressed by rVV and rVSV with OT-I.SJL cells undergoing three rounds of division in response to rVV-OVA and two rounds of division in response to rVSV-OVA (Figure 4.4B and 4.4D).

The majority of antigens presented on MHC class I are transported into the ER via the Transporter Associated with Antigen Processing (TAP) (Androlewicz et al., 1993; Heemels et al., 1993; Neefjes et al., 1993). *In vitro* experiments using OVA have demonstrated that MHC class I presentation of OVA is TAP-dependent (Norbury et al., 2001). Yet, Norbury et al demonstrated that the presentation of OVA expressed from rVV was TAP-independent *in vivo* (Norbury et al., 2001). Thus, to determine whether the viral vector expressing OVA affects the antigen processing and presentation pathways available to OVA, we adoptively transferred OT-I.SJL lymphocytes into TAP-/- mice. Cells lacking TAP are extremely inefficient at presenting antigen on MHC class I; initial experiments with the optimal doses of rVV-OVA and rVSV-OVA

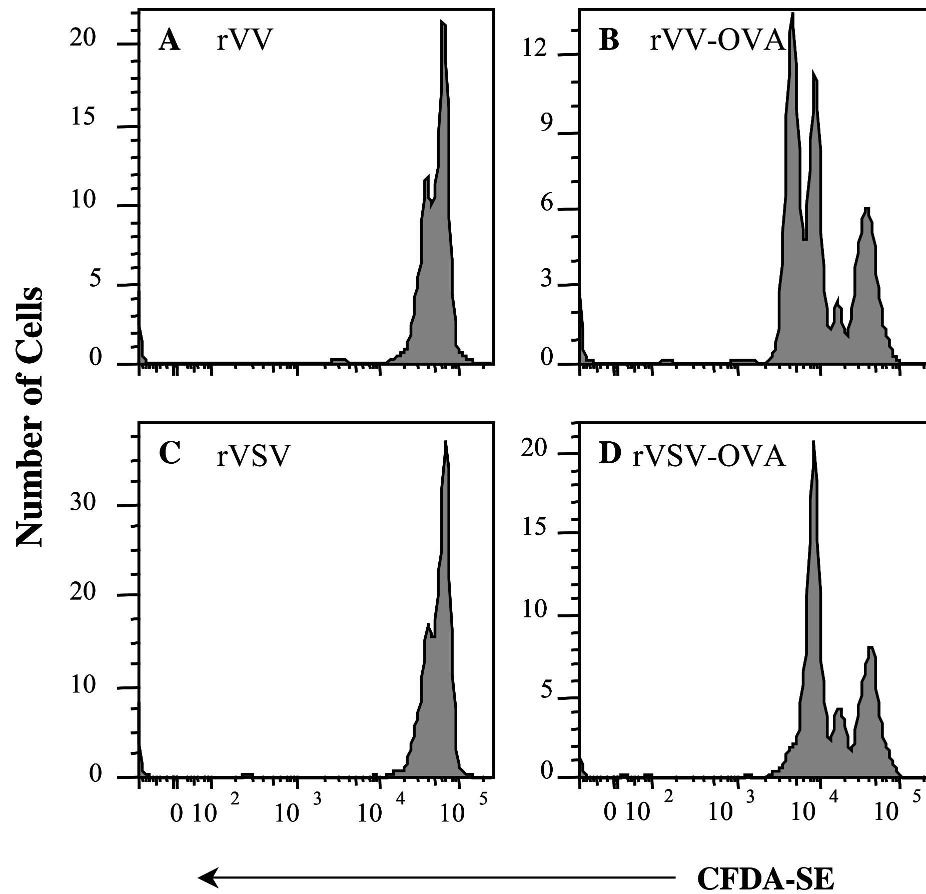

Figure 4.4 CD8+ Immune Response in wild type mice.

B6 mice that had received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells were immunized with $2.5x10^6$PFU rVV (A), $2.5x10^6$PFU rVV-OVA (B), $2.5x10^5$PFU rVSV (C), or $2.5x10^5$PFU rVSV-OVA (D). Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Figure is representative of 3 experiments.

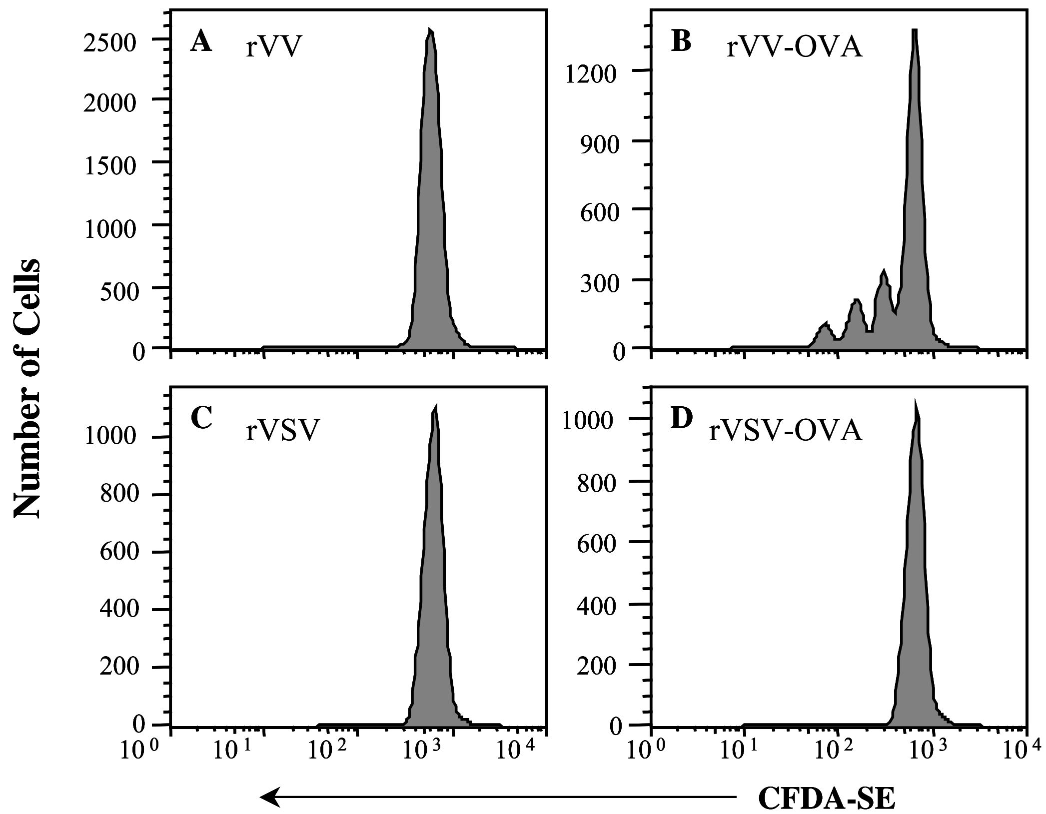

Figure 4.5 CD8 Immune Response in TAP-/- Mice.

TAP-/- mice that had been gamma-irradiated two days prior, received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells followed by immunization with $1x10^8$PFU rVV (A), rVV-OVA (B), rVSV (C) or rVSV-OVA (D). Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Figure is representative of 3 experiments.

produced no detectable activation of OT-I.SJL T cells in TAP-/- mice. Thus, we chose to infect TAP-/- mice with the maximum dose of virus available, $1x10^8$PFU/mouse. Proliferation of the adoptively transferred OT-I.SJL cells was then used as a measure of CD8+ T cell activation. OT-I.SJL cell proliferation was observed in TAP-/- mice infected with rVV-OVA, but not in mice infected with rVSV-OVA (Figure 4.5), indicating that the presentation of OVA expressed from rVSV, but not rVV, was TAP-dependent.

4.2.5 CD8+ T cell Response *In Vitro*

To determine whether the CD8+ T cell response we observed *in vivo* in TAP-/- mice was due to direct presentation from rVV-OVA infected cells, it was necessary to use an *in vitro* system. Use of an *in vitro* system is necessary in order to eliminate the cross-priming pathway. Thus, wild-type and TAP-/- fibroblasts were infected with rVV-OVA or rVSV-OVA. Instead of using OT-I.SJL cells to measure the ability of the infected cells to stimulate CD8+ T cells, we used the K^b-restricted $OVA_{257-264}$-specific B3Z hybridoma. B3Z produce β gal upon activation, allowing activation to be measured by staining with the β gal substrate X-Gal. Fibroblasts were infected with rVV-OVA or rVSV-OVA and cultured with B3Z cells overnight prior to X-gal staining. Uninfected fibroblasts as well as fibroblasts infected with the parent viral vectors were included as well as negative controls. Neither rVV-OVA nor rVSV-OVA infected TAP-/- fibroblasts were able to activate B3Z cells, indicating that the presentation of OVA from infected fibroblasts *in vitro* is TAP-dependent (Figure 4.6A). It is conceivable that TAP-independent presentation of virally expressed OVA may require professional antigen presenting cells. Thus, we examined the ability of infected wild type and TAP-/- bone marrow dendritic cells (BMDC) to activate B3Zs. Similar to the observations with infected fibroblasts, neither rVV-OVA nor

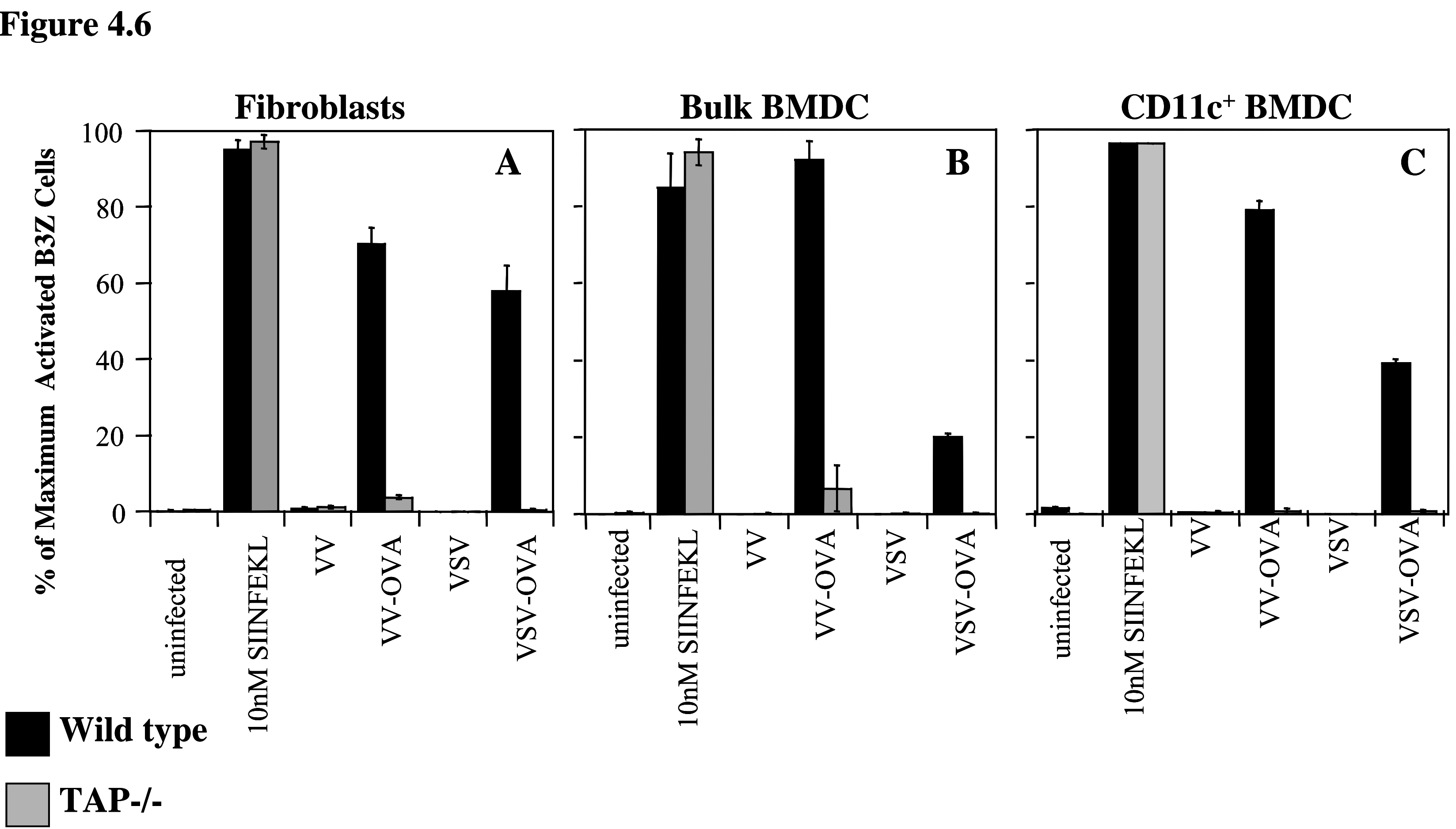

Figure 4.6
Fibroblasts
Bulk BMDC
CD11c+ BMDC
A
B
C
% of Maximum Activated B3Z Cells
100
80
60
40
20
0
uninfected
10nM SIINFEKL
VV
VV-OVA
VSV
VSV-OVA
Wild type
TAP-/-

Figure 4.6 OVA is TAP dependent *in vitro*.

Wild type (black bars) or TAP-/- (gray bars) were infected as indicated with an MOI=10 for four hours and then used to stimulate the $OVA_{257-264}$ specific hybridoma B3Z. Cells were co-cultured overnight and then stained with X-gal. (A) Fibroblasts, (B) Bulk BMDC, (C) BMDC purified for CD11c. Each panel represents 3 independent experiments; errors bars show ±SEM.

rVSV-OVA infected TAP-/- fibroblasts were able to activate B3Z cells (Figure 4.6B). rVV-OVA infected wild type BMDC activated considerably more B3Zs than rVSV-OVA infected wild type BMDC (Figure 4.6B), suggesting that either rVV-OVA infects a greater percentage BMDC than rVSV-OVA or that the higher concentration of OVA produced by rVV-OVA infection (Figure 4.3) enhances activation of B3Z cells. Later experiments determined that 10.4 percent of BMDC were infected by rVSV-OVA while 32.6 percent of BMDC were infected (Figure 5.8). However, neither rVV-OVA infected nor rVSV-OVA infected TAP-/- BMDC were able to activate B3Z cells (Figure 4.6B). We then repeated the experiment using BMDC purified for CD11c, a dendritic cell marker, in an attempt to enhance the presentation of OVA. Although the activation of B3Zs by rVSV-OVA was enhanced when wild type purified BMDC were used (Figure 4.6B and C), the purified TAP-/- BMDC yielded results identical to those of bulk TAP-/-BMDC (Figure 4.6B and C). Contrary to the *in vivo* results, infected TAP-/- cells were unable to activate the B3Zs, indicating that the presentation of OVA *in vitro* is TAP-dependent (Figure 4.6), suggesting that CD8+ T cell response to rVV-OVA in TAP-/- mice was due to TAP-independent cross-priming of OVA expressed by rVV. TAP-/- BMDC or fibroblasts pulsed with $OVA_{257-264}$ as a positive control were able to activate B3Z cells, indicating that the inability of the infected TAP-/- cells to activate B3Z cells was due to a lack of TAP rather than a lack of MHC class I (Figure 4.6). Since our viral stocks, even when purified, contain significant amounts of OVA (Figure 4.7A), we incubated rVV-OVA and rVSV-OVA with neutralizing antibodies prior to co-culture with BMDC and B3Zs to determine what extent of the B3Z response was due to OVA present in the viral stocks. No B3Z activation was seen in response to rVV-OVA or rVSV-OVA in the presence of neutralizing antibodies, indicating that our results are due to infection of the fibroblasts and BMDC rather than to OVA present in the viral stocks (Figure 4.7B). Together these results indicate that the differing requirement for TAP demonstrated *in vivo* can not be reproduced *in vitro*, preventing us from further characterizing the antigen processing pathways

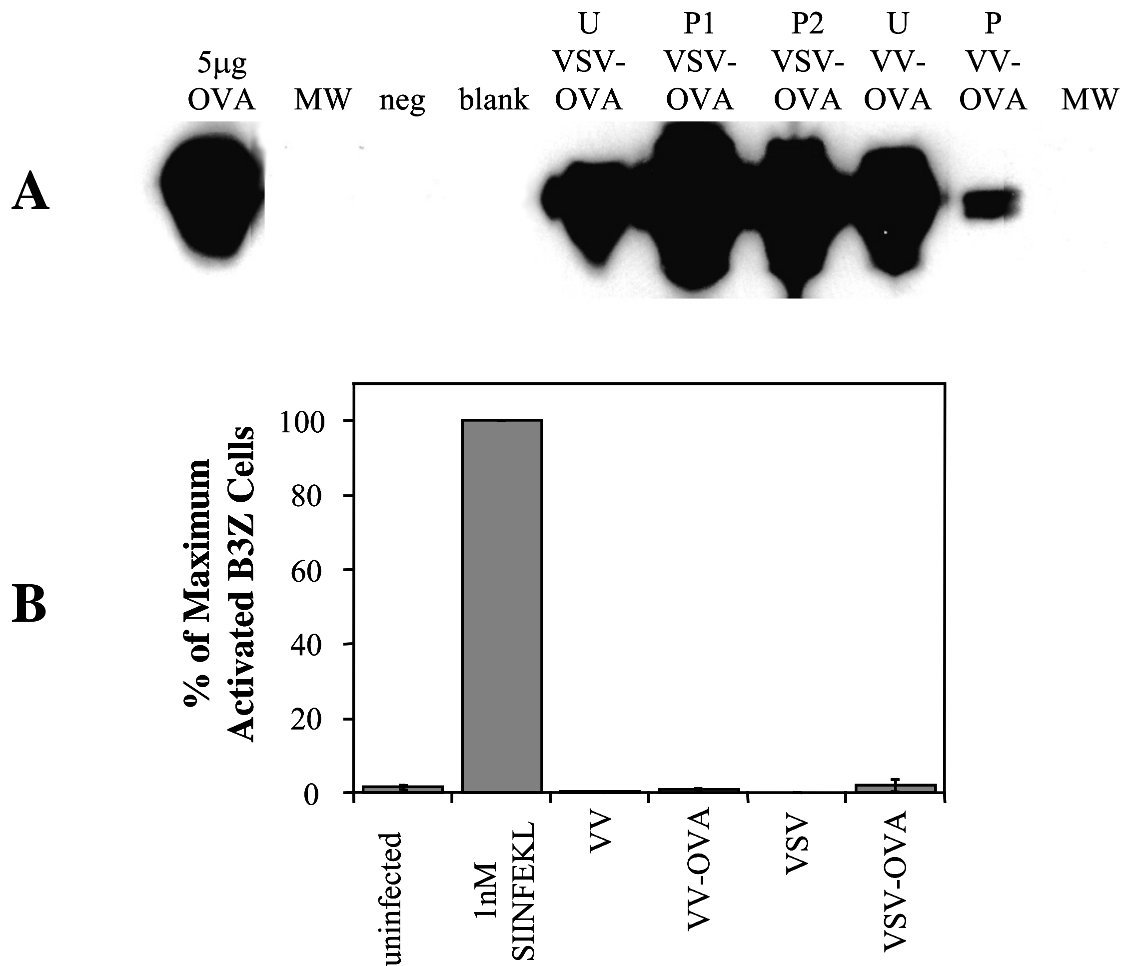

Figure 4.7 B3Z are not activated by OVA present in viral stocks.

(A) Western blot of unpurified and purified viral stocks. $1.5x10^6$ PFU of unpurified and purified rVV-OVA or rVSV-OVA was loaded onto gels and then blotted for OVA. U indicates the unpurified virus while P indicates purified virus. The number following P indicates the number of rounds of purification. Gel is representative of two experiments. (B) Wild type bulk BMDC were infected with $1x10^7$ PFU of antibody-neutralized virus for four hours and then used to stimulate the $OVA_{257-264}$ specific hybridoma B3Z. Cells were co-cultured overnight and then stained with X-gal. Panel represents 3 independent experiments; errors bars show ±SEM.

Figure 4.8

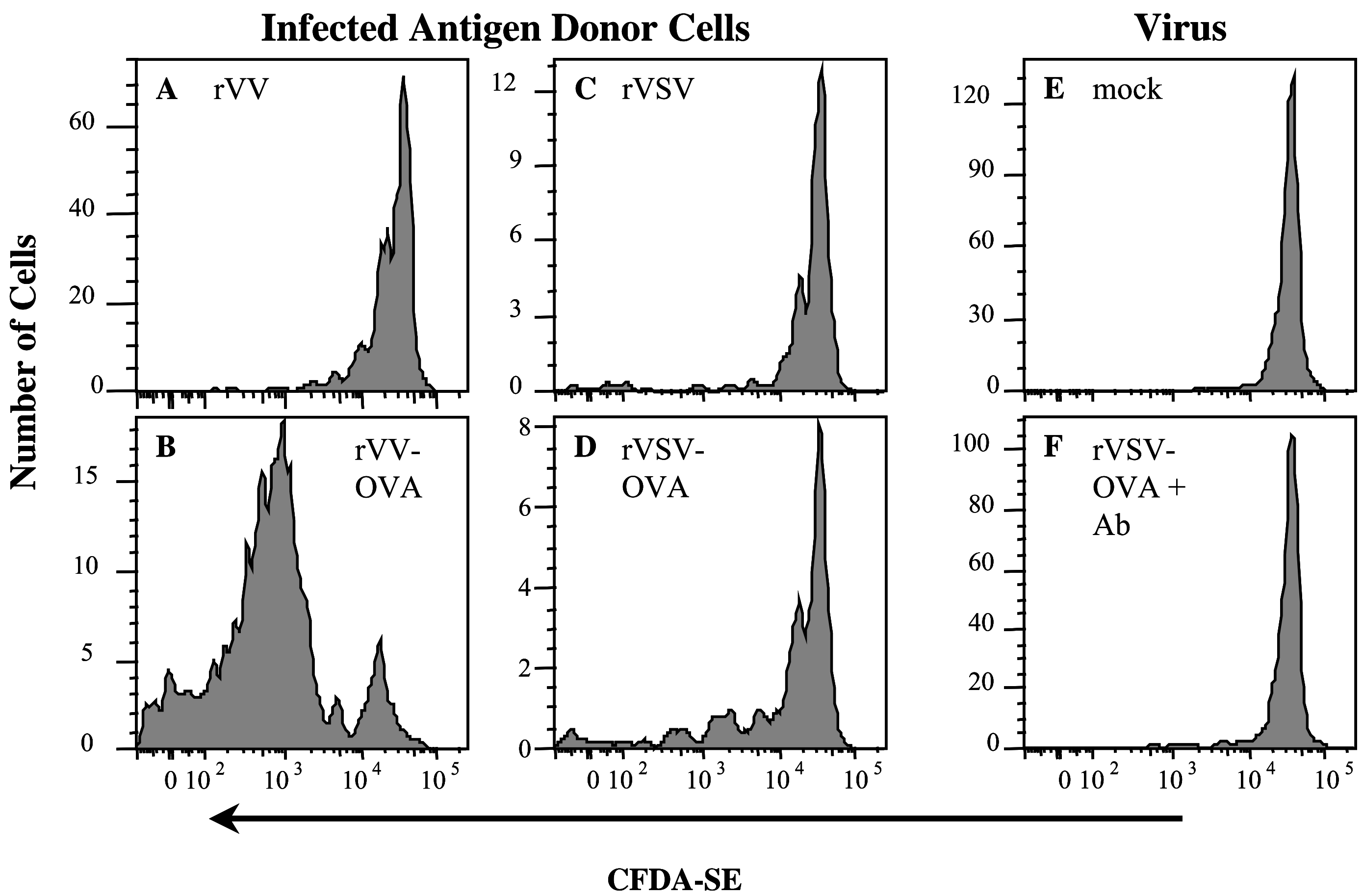

Figure 4.8 Ability of rVV and rVSV expressed OVA to induce cross-priming in wild-type mice.

B6 mice received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells followed by immunization with rVV (A), rVV-OVA (B), rVSV (C), or rVSV-OVA (D) infected β_2-microglobulin negative cells. To demonstrate the effectiveness of neutralizing antibody treatment on VSV, mice were also immunized with HBSS (E) or $1x10^7$PFU VSV-OVA treated with neutralizing antibody (F). Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Each panel is representative of 3 experiments.

available to virally expressed OVA and further suggesting that the *in vivo* results are due to TAP-independent cross-priming of OVA expressed by rVV.

4.2.6 Ability of rVV-OVA and rVSV-OVA to Induce Cross-Priming *In Vivo*

Norbury *et al.* previously demonstrated that the rVV expressed OVA was TAP-independent *in vivo* due to TAP-independent cross-priming (Norbury et al., 2001). Therefore, we decided to examine whether rVSV-OVA could induce cross-priming *in vivo*. β_2-microglobulin negative cells, which are unable to directly present antigen (Norbury et al., 2004), were infected with rVV-OVA or rVSV-OVA for four hours and then treated with psoralen and ultraviolet (UV) irradiation (Tsung et al., 1996) (Figure 3.1) to prevent viral spread from the infected cells to the recipient mouse. Psoralen and UVC treatment does not completely prevent VSV replication, so these cells were also treated with neutralizing antibody (Figure 4.8E and F). Infected cells were then introduced intraperitoneally (IP) into B6 mice or TAP-/- mice that had previously received OT-I.SJL lymphocytes. Three days later, proliferation of the adoptively transferred OT-I.SJL cells was used as a measure of CD8+ T cell activation. OT-I.SJL cells proliferated in response to both rVV-OVA infected cells as well as in response to rVSV-OVA infected cells, indicating that both rVV and rVSV expressed OVA could be cross-primed in wild type mice (Figure 4.8). In wild type mice, rVSV-expressed OVA stimulated less cross-priming than rVV-expressed OVA, indicating that rVV-expressed OVA may more readily access the cross-priming pathway. However, low levels of cross-priming were observed for rVSV-OVA in all three experiments, with Figure 4.8D representing the average amount of proliferation observed in response to rVSV-OVA infected β_2-microglobulin negative cells. Only rVV expressed OVA was able to induce proliferation of OT-I.SJL cells in TAP-/- mice (Figure 4.9), indicating that rVV-expressed OVA is cross-primed in a TAP-independent manner.

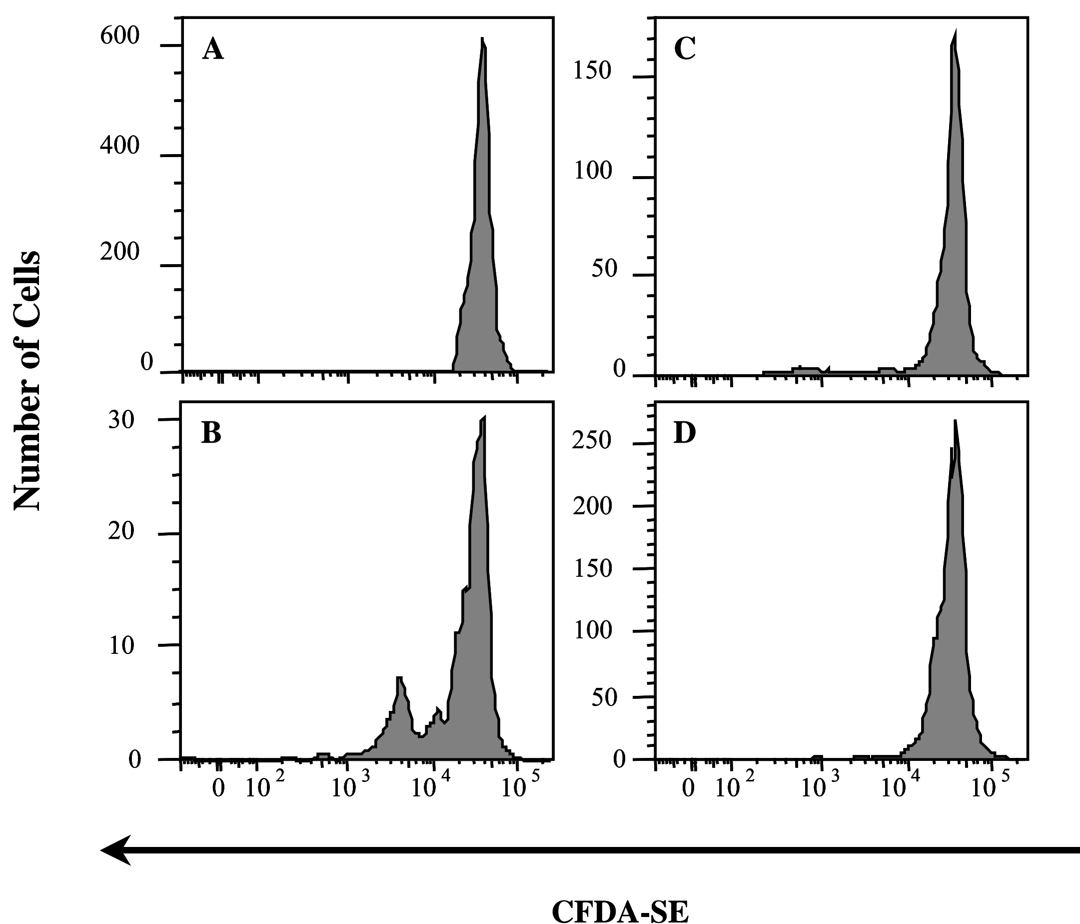

Figure 4.9 Ability of rVV and rVSV expressed OVA to induce cross-priming in TAP-/- mice.

TAP-/- mice that had been gamma-irradiated two days prior received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells followed by immunization with rVV (A), rVV-OVA (B), rVSV (C), or rVSV-OVA (D) infected β_2-microglobulin negative cells. Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Each panel is representative of a single experiment.

4.2.7 Reconstituting Cross-Priming in TAP-/- Mice

The observation that rVV-expressed OVA, but not rVSV-expressed OVA, could be cross-primed in a TAP-independent manner led us to investigate the effect of virus infection on the ability of OVA to be cross-primed. To investigate the effect of viral infection, β_2-microglobulin negative cells were electroporated with OVA either prior to or after 4-hour infection with either rVV or rVSV. Previous studies have used cells electroporated with OVA to induce cross-priming (Donohue et al., 2006). Infected cells were then treated with psoralen and UV irradiation to prevent viral spread from the infected cells to the recipient mouse. VSV infected cells were also treated with neutralizing antibody as above. Infected cells were then introduced IP into wild type B6 mice that had previously received OT-I.SJL lymphocytes. Three days later, proliferation of the adoptively transferred OT-I.SJL cells was used as a measure of CD8+ T cell activation. All cells electroporated with OVA were able to induce cross-priming in B6 mice regardless of infection type (uninfected, rVV infected or rVSV infected) or the time point of OVA electroporation (either before or after infection), demonstrating that we could reconstitute cross-priming in this manner (Figure 4.10). The time point of infection did not affect the ability of OVA electroporated cells to cross-prime, hence we decided to only electroporate OVA prior to infection in TAP-/- mice. β_2-microglobulin negative cells were electroporated with OVA and treated as above before being injected IP into TAP-/- mice that had previously received an adoptive transfer of OT-I.SJL lymphocytes. Proliferation of the adoptively transferred OT-I.SJL cells was used as a measure of CD8+ T cell activation three days post-immunization. Surprisingly, neither uninfected nor rVSV infected OVA electroporated cells were able to stimulate cross-priming in TAP-/- mice, yet rVV infected OVA electroporated cells were (Figure 4.11). This indicates that rVV infection somehow enhances TAP-independent cross-priming of OVA.

Figure 4.10

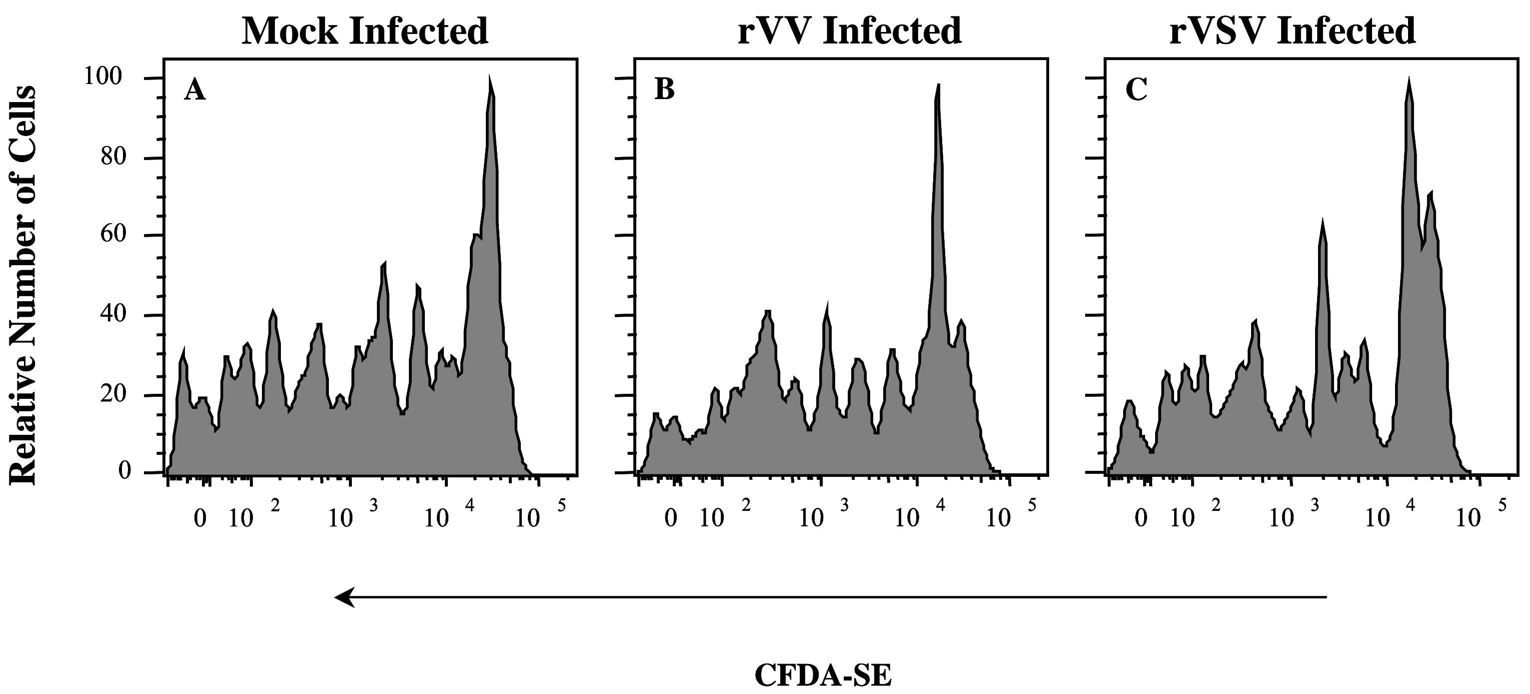

Figure 4.10 Reconstitution of cross-priming in wild type mice using OVA electroporated cells.

B6 mice received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells followed by immunization with uninfected (A), rVV infected (B) or rVSV infected (C), OVA electroporated β_2-microglobulin negative cells. Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Figure is representative of 2 experiments.

Figure 4.11

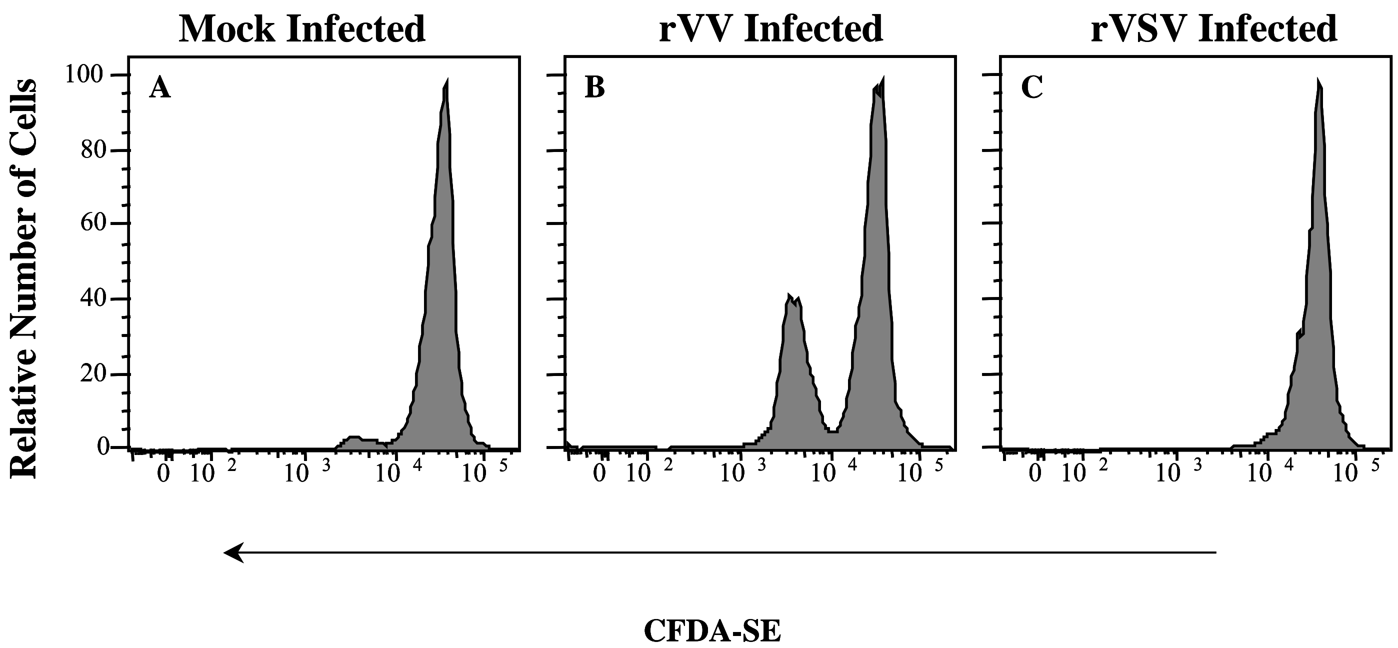

Figure 4.11 rVV permits TAP-independent cross-priming of OVA from electroporated cells.

TAP-/- mice that had been gamma-irradiated two days prior received an adoptive transfer of CFDA-SE-labeled OT-I.SJL cells followed by immunization with uninfected (A), rVV infected (B), or rVSV infected (C), OVA electroporated β_2-microglobulin negative cells. Three days later CD8+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Panels represent a single experiment.

4.3 Discussion

Rational design of vaccines to stimulate T cell mediated immunity requires not only an understanding of the antigen processing and presentation pathways but how the viral vector used to express the antigen affects the ability of the antigen to access antigen processing and presentation pathways. In this study, we set out to characterize how the viral vector used to express the antigen affects the antigen processing and presentation pathways available to the antigen using rVV and rVSV as our viral vectors to express the model antigen OVA. Our study began by characterizing the primary CD8+ T cell response to OVA in wild type mice. Thus, wild type mice were immunized with the optimal dose of virus and both the virus-specific and the OVA-specific CD8+ T cell response were measured by ICS for IFN-γ six days after immunization. OVA expressed by both rVV and rVSV stimulated naïve CD8+ T cells to produce IFN-γ (Figure 4.2). However, rVSV-expressed OVA was better at stimulating OVA-specific naïve CD8+ T cells to produce IFN-γ than rVV-expressed OVA (Figure 4.2), with approximately 5 times more cells producing IFN-γ in response to rVSV-expressed OVA than rVV-expressed OVA. This enhanced response to rVSV-expressed OVA is particularly interesting as the optimal dose of rVSV-OVA is 10-fold less than that of rVV-OVA (Figure 4.1), indicating that it takes a smaller dose of virus to stimulate more OVA-specific CD8+ T cells. The difference in the response to OVA expressed by the viral vectors could be explained by differences in the expression level of OVA, differences in the secretion of OVA or differences in the antigen processing and presentation pathways available to OVA.

Thus, we next determined both the protein expression levels as well as the secretion of OVA expressed from the two viral vectors by Western blot. Western blot results demonstrated that expression of OVA from rVV-OVA was at least one thousand-fold higher than expression of OVA by rVSV-OVA (Figure 4.3), as we had to dilute fibroblasts infected with rVV-OVA one

thousand-fold before loading them on a gel. CD8+ T cells have been demonstrated to have a restricted intensity of activation signal (Alexander-Miller et al., 1996; Kroger and Alexander-Miller, 2007; Rocha et al., 1995). Thus, the increased level of OVA from rVV-OVA could have surpassed the activation signal of the CD8+ T cells, resulting in tolerance to OVA or in deletion of the CD8+ T cell, explaining the results to rVV-expressed OVA in naïve wild type mice. Although OVA is a secreted protein (Braell and Lodish, 1982; Meek et al., 1982; Palmiter et al., 1978), OVA was demonstrated to be sequestered within rVV-OVA infected cells, but secreted from rVSV-OVA infected cells. While secretion of OVA from rVSV-OVA-infected fibroblasts is demonstrated by treating the infected cells with BFA, a vesicular trafficking inhibitor, it is possible that blebbing releases some of the OVA produced by rVSV-OVA-infected cells. Future experiments could address OVA being released by blebbing by centrifuging media from infected cells. Additionally, secretion of OVA could also be addressed by determining if BFA treatment prevents OVA from appearing in the supernatant. This difference in the secretion status could also explain the increased OVA-specific CD8+ T cell response to rVSV-OVA infection.

With differences in both the protein expression levels and the secretion of OVA expressed from the viral vectors, we turned our attention to the antigen processing and presentation pathways available to OVA expressed by the viral vectors *in vivo*. Despite the difference in stimulating naïve CD8+ T cells, both rVV-OVA and rVSV-OVA stimulated OVA-specific transgenic CD8+ T cells to undergo similar levels of proliferation in wild type B6 mice (Figure 4.4). In TAP-/- mice, however, only OVA expressed by rVV was capable of stimulating proliferation in OVA-specific CD8+ transgenic T cells, implying either that the TAP-independent pathway of MHC class I processing was unavailable to rVSV-expressed OVA or that rVV infection enabled OVA to access the TAP-independent pathway (Figure 4.5). The discovery that rVV enabled OVA to access the TAP-independent pathway led us to consider which cells were actually presenting OVA in this TAP-independent manner.

To eliminate contributions from the cross-priming pathway, an *in vitro* system was used to determine whether virally infected cells were directly presenting OVA. Despite infecting three different cell types, including fibroblasts, BMDC and CD11c+ BMDC, all of our *in vitro* experiments demonstrated that the presentation of OVA was TAP-dependent (Figure 4.6), regardless of the virus used to express it. The TAP-dependency of our *in vitro* results were not due to an inability of the TAP-/- cells to present antigen, as the TAP-/- cells pulsed with the $OVA_{257-264}$ peptide as a positive control were able to stimulate the OVA-specific CD8+ T cell hybridomas. Thus, these results indicate that the direct presentation of OVA is TAP-dependent, suggesting that the TAP-independent presentation of rVV-expressed OVA observed *in vivo* was due to TAP-independent cross-priming.

Norbury *et al.* had previously demonstrated that rVV-expressed OVA was TAP-independent *in vivo* due to TAP-independent cross-priming (Norbury et al., 2001). Thus, we examined the ability of rVV- and rVSV-expressed OVA to be cross-primed *in vivo*. Both rVV-OVA and rVSV-OVA infected β_2-microglobulin negative cells were able to induce cross-priming in wild type B6 mice (Figure 4.8), although rVV-OVA elicited approximately twenty times more cells to proliferate than rVSV-OVA. In TAP-/- mice, however, only rVV-OVA infected β_2-microglobulin negative cells were able to stimulate cross-priming (Figure 4.9), implying that rVV expression of OVA permitted TAP-independent cross-priming. The increased number of transgenic CD8+ T cells proliferating in response to rVV-OVA infection as compared to rVSV-OVA infection in B6 mice may reflect additional TAP-independent pathway(s) available to rVV expressed OVA (Figure 4.8). However, it should be noted that in the absence of OVA-specific transgenic CD8+ T cells, OVA expressed by rVSV is better at stimulating CD8+ T cells than OVA expressed by rVV (Figure 4.2).

The dependence on TAP for the presentation of rVSV-expressed OVA may be due to either the expression of OVA from VSV or it may be due to some intrinsic effect of VSV

infection on the antigen donor cell. Alternatively, some intrinsic effect of VV infection could enhance the antigen processing pathways available to OVA. To examine whether virus infection has some intrinsic effect on the antigen donor cell, we infected β_2-microglobulin negative cells previously electroporated with OVA with rVSV or rVV. If virus infection has an intrinsic effect on the antigen donor cell, we would expect to see an inhibition of cross-priming under these circumstances. We first demonstrated that cross-priming could be reconstituted in this fashion in wild type B6 mice (Figure 4.10). We then sought to examine whether cross-priming in TAP-/- mice could also be reconstituted. Surprisingly, despite the fact that all cells were electroporated with identical amounts of OVA, we found that only rVV infected β_2-microglobulin negative cells, not uninfected nor rVSV infected, could stimulate TAP independent cross-priming (Figure 4.11). This result implies that rVSV infection does not inhibit TAP-independent cross-priming, because uninfected, OVA-electroporated cells could not stimulate TAP-independent cross-priming (Figure 4.11A). Furthermore, these results imply that rVV infection enhances TAP-independent cross-priming, allowing OVA to access an antigen processing pathway that it would otherwise be excluded from.

From our studies, we know that rVV-OVA expresses one thousand-fold more OVA than rVSV-OVA and that rVV-expressed OVA is sequestered within the cell while rVSV-expressed OVA is secreted. Previous studies have demonstrated that *in vivo* cross-priming uses long-lived stable protein (Donohue et al., 2006; Norbury et al., 2004; Wolkers et al., 2004). Conceivably, the increased OVA expression and the sequestration within the cells would enhance the ability of rVV-expressed OVA to access the cross-priming pathway. However, the potentially increased level of rVV-expressed OVA available to the cross-priming pathway does not explain how rVV-expressed OVA accesses the TAP-independent cross-priming pathway. Further studies with other antigens may elucidate the mechanism by which rVV conveys access to the TAP-independent pathway.

Our studies demonstrate that the viral vector does influence the ability of an antigen to access antigen processing and presentation pathways. Whether the ability of the antigen to access certain antigen processing and presentation pathways will affect the secondary immune response remains to be determined. While rVV may appear to be the better vaccine vector because it permits the antigen to access the TAP-independent cross-priming pathway (Figures 4.9 and 4.11), rVSV stimulated five times more CD8+ T cells in naïve mice (Figure 4.2). Previous studies have demonstrated the importance of the number of T cells stimulated in the primary immune response on the memory T cell response (Hou et al., 1994; Kundig et al., 1996). Thus, the greater number of CD8+ T cells stimulated by rVSV could be important in establishing long-term protective immunity, something not addressed by this study. Future studies should examine not only the antigen processing and presentation pathways available to virally-expressed antigens, but also what those antigen processing and presentation pathways mean to the long-term protective immunity to the virally-expressed antigen.

Chapter 5

Effect of Viral Vectors on Stimulating a CD4+ T cell Response to a Model Antigen

5.1 Introduction

Infectious diseases that currently pose the greatest threat to humans, AIDS, tuberculosis and malaria, are likely to require a vaccine strategy designed to elicit T cell-mediated immunity. Recombinant viral vectors expressing transgenes from other diseases have been proposed as a means of stimulating T cell immunity. While studies have examined the ability of transgenes expressed by recombinant viruses to stimulate T cells (Cooney et al., 1991; Haglund et al., 2002; Konishi et al., 1998; Natuk et al., 2006; Ockenhouse et al., 1998; Rose et al., 2001; Sereinig et al., 2006), few studies have systematically examined the antigen processing pathways available to a transgene when expressed by different viral vectors. Our goal is to examine the MHC class II antigen processing pathways available to a model antigen, ovalbumin (OVA), when expressed by either recombinant vaccinia virus (rVV) or recombinant vesicular stomatitis virus (rVSV), two of the recombinant viral vectors often proposed as vaccine vectors.

Many studies have utilized rVV or rVSV as vaccine vectors to stimulate T cell immunity to a transgene (Perkus et al., 1991; Perkus et al., 1985). The advantage to using these viruses as vaccine vectors is that they are both able to accept large transgenes, at least 25kb for rVV (Schnell et al., 1996) and 2.5kb for rVSV (Hosken et al., 1995; Robertson et al., 2000; Rush et al., 2002); and are able to be grown to high titer. In this study, we will use rVV and rVSV to express a model antigen, ovalbumin (OVA). Unlike many viral proteins used as model antigens, expression of OVA will not exert an effect on the virus used to express it. Thus, any observed

differences will be due to the virus used to express OVA, rather than an effect of OVA on the vaccine vector. In addition, the CD4+ T cell response to OVA has been extensively characterized (Barnden et al., 1998) and we can use transgenic mice that express a transgenic T cell receptor specific for $OVA_{323-339}$ complexed to $I\text{-}A^b$ (Cassell and Forman, 1988; Fernando et al., 2002; Husmann and Bevan, 1988; Keene and Forman, 1982; Shedlock and Shen, 2003; Sun and Bevan, 2003).

CD4+ T cells have been demonstrated to be required for the establishment of CD8+ T cell memory responses (Buller et al., 1987; Rahemtulla et al., 1991; Wu and Liu, 1994), particularly in the establishment of CD8+ T cell memory responses to certain acute viral infections (Burkhart et al., 1994b; Leist et al., 1987; Littaua et al., 1992; Xu et al., 2004). Thus, activation of CD4+ T cells is of particular interest to vaccine strategies designed to stimulate protective CD8+ T cell responses. Both vaccinia virus (VV) and vesicular stomatitis virus (VSV) infection stimulate potent CD4+ T cell responses, which are required for the antibody-mediated clearance of both viruses (Crotty et al., 2003; Hammarlund et al., 2003; Littaua et al., 1992). In VV-immunized humans, VV-specific CD4+ T cells were detected after a single immunization and were still detected up to 75 years post-vaccination (Burkhart et al., 1994b; Leist et al., 1987). VSV-specific CD4+ T cells were easily detected post-immunization, but no studies have investigated how long such cells linger post-infection (Thomsen et al., 1997). Adoptive transfer of VSV-specific CD4+ T cells into T cell-deficient mice was sufficient to prevent the T cell deficient mice from succumbing to a subsequent lethal VSV challenge (Andersen et al., 1999; Thomsen et al., 1997; Xu et al., 2004), emphasizing the importance of CD4+ T cell help in mediating VSV infection. Neither VV-specific nor VSV-specific CD4+ T cells were required for the establishment of the primary virus-specific CD8+ T cell responses (Xu et al., 2004). VV-specific CD4+ T cells, however, were required for the establishment of VV-specific memory CD8+ T cell responses in mice (Falk et al., 1994; Rudensky et al., 1992; Rudensky et al., 1991).

CD4+ T cells recognize peptides presented on MHC class II molecules. MHC class II molecules have open-ended peptide binding grooves which permit the binding of peptides that are at least 13 amino acid residues long and can be longer (Castellino et al., 1998; Grewal et al., 1995; Villadangos et al., 2000). Peptides presented on MHC class II are derived from exogenous (Mouritsen et al., 1994; Newcomb and Cresswell, 1993; Rudensky et al., 1991) or endogenous proteins (Roche and Cresswell, 1990; Teyton et al., 1990) processed in the endosomal compartments. Previous studies have demonstrated that the antigen processing and presentation pathways available to ovalbumin (OVA) for presentation on MHC class I are dependent on the virus used to express OVA (Chapter 4). While running controls for the MHC class I processing experiments, transporter-associated with antigen processing (TAP)-dependent MHC class II presentation of OVA was observed. TAP, which transports poly-peptides into the ER for presentation on MHC class I, is not typically thought to be involved in MHC class II processing as MHC class II molecules are typically prevented from binding peptides in the ER by the invariant chain (Blum and Cresswell, 1988; Nowell and Quaranta, 1985). The invariant chain blocks the peptide binding groove of MHC class II molecules until it is cleaved off in endolysosomal compartments (Carmichael et al., 1996; Malnati et al., 1992; Tewari et al., 2005). However, prior to binding the invariant chain, nascent MHC class II molecules could potentially interact with and bind peptides present in the ER. Several groups have reported a requirement for TAP in the transport of cytosolic-processed MHC class II peptides (Barnden et al., 1998; Burkhart et al., 1994a; Tewari et al., 2005). Based on these and our own observation, we examined the MHC class II processing pathways available to OVA when expressed by recombinant vaccinia virus (rVV) or by recombinant vesicular stomatitis virus (rVSV).

5.2 Results

5.2.1 MHC Class II Processing Pathways Available *In Vivo* to Virally Expressed OVA

While running controls for the experiments described in Chapter 4, TAP-dependent MHC class II processing of virally expressed OVA was observed. In order to evaluate this observation, it was first necessary to examine the CD4+ T cell response in relation to antigen presentation. This required the use of T cells from transgenic mice, OT-II mice, that express a transgenic T cell receptor specific for the CD4+ T cell determinant $OVA_{323-339}$ complexed to I-A^b (Pape et al., 1997). Adoptive transfer of transgenic T cells specific for OVA results in a high frequency of CD4+ T cells specific for OVA (Malnati et al., 1992; Tewari et al., 2005). The high frequency of OVA-specific CD4+ T cells permits examination of the CD4+ T cell response in relation to antigen presentation without having to wait for several rounds of division and acquisition of effector function in order to detect MHC class II presentation. Thus, MHC class II presentation can be detected by the activation and proliferation of the adoptively transferred OT-II cells. Additionally, by crossing the OT-II mice with B6.SJL mice (OT-II.SJL), the adoptively transferred cells can be identified by staining for the CD45.1 marker, which is expressed only by the transferred cells but not the recipient wild type B6 or TAP-/- mice. Prior to examining the MHC class II presentation pathways available to virally expressed OVA, it is necessary to examine the proliferation response of the OT-II.SJL cells in wild type mice. Lymphocytes harvested from OT-II.SJL mice were thus adoptively transferred into wild-type mice. Mice that received adoptive transfers were subsequently infected with the optimal dose ($2.5x10^6$ PFU/mouse for rVV and $2.5x10^5$ PFU/mouse for rVSV) of virus as measured in Figure 4.1. Mice were infected with either rVV-OVA or rVSV-OVA. rVV and rVSV infected mice were included as negative controls. Proliferation of adoptively transferred OT-II.SJL cells was then used as a

measure of CD4+ T cell activation (Figure 5.1). OT-II.SJL proliferation was similar in response to both rVV-OVA and rVSV-OVA (Figure 5.1B and D). To evaluate the TAP-dependence of MHC class II processing of virally expressed OVA, we adoptively transferred OT-II.SJL cells into TAP-/- mice. As our initial observation was from a control of the MHC class I processing experiments in TAP-/- mice (Chapter 4), we chose to use the same dose of virus as was used in our initial experiments, $1x10^{8}$PFU/mouse. As in the experiment above, proliferation of OT-II.SJL cells was then used as an indication of MHC class II presentation. OT-II.SJL cell proliferation was observed in TAP-/- mice infected with rVV-OVA, but not in TAP-/- mice infected with rVSV-OVA (Figure 5.2), indicating that the presentation of OVA expressed from rVSV, but not rVV, was TAP-dependent.

5.2.2 Ability of Infected Cells to Present Virally Expressed OVA on MHC Class II

Previous studies reporting TAP-dependent presentation of antigens on MHC class II have utilized endogenously expressed antigens(Black et al., 2002). To determine whether the TAP-dependency we observed *in vivo* was due to the virally infected cell presenting OVA on MHC class II or whether it was due to acquisition of OVA from other virally infected cells, it was necessary to use an *in vitro* system. We first tried infecting APC to determine whether they could present virally-expressed OVA to *ex vivo* OT-II.SJL cells. *Ex vivo* OT-II.SJL cells are isolated from mouse spleens and lymph nodes and used immediately, without stimulation or culture. Thus, *ex vivo* OT-II.SJL cells would be expected to respond as naïve T cells rather than as cultured cells. Wild type and TAP-/- bone marrow dendritic cells (BMDC) were used as APC. BMDC were infected with rVV-OVA or rVSV-OVA. Proliferation of *ex vivo* OT-II.SJL cells was used to measure the ability of infected BMDC to present to CD4+ T cells. Uninfected BMDC as well as BMDC infected with the parent viral vectors were included as negative

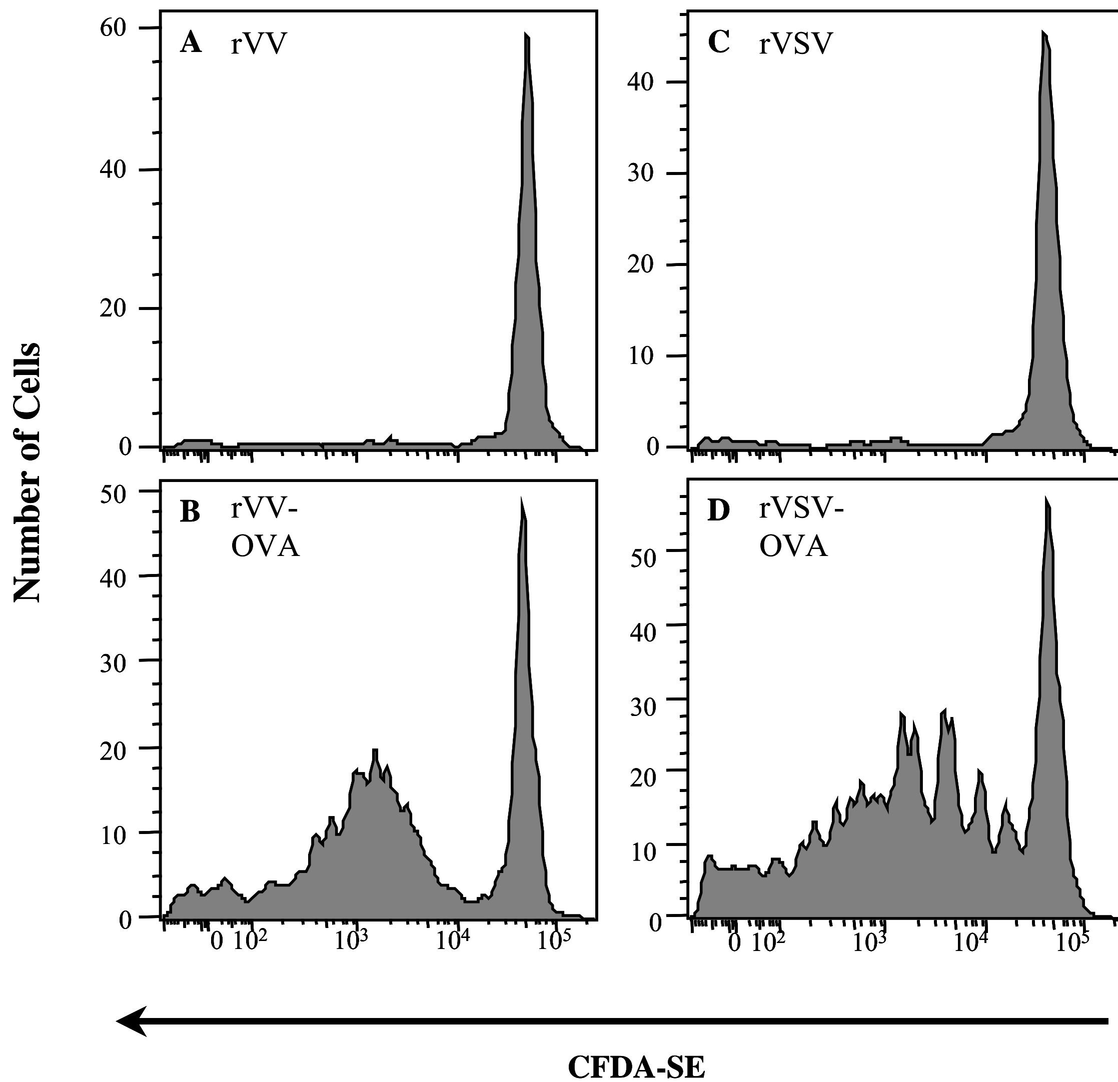

Figure 5.1 CD4+ T cell Response in wild type mice.

B6 mice that had received an adoptive transfer of CFDA-SE-labeled OT-II.SJL cells were immunized with $2.5x10^6$PFU rVV (A), $2.5x10^6$PFU rVV-OVA (B), $2.5x10^5$PFU rVSV (C), or $2.5x10^5$PFU rVSV-OVA (D). Three days later CD4+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Figure is representative of 5 experiments.

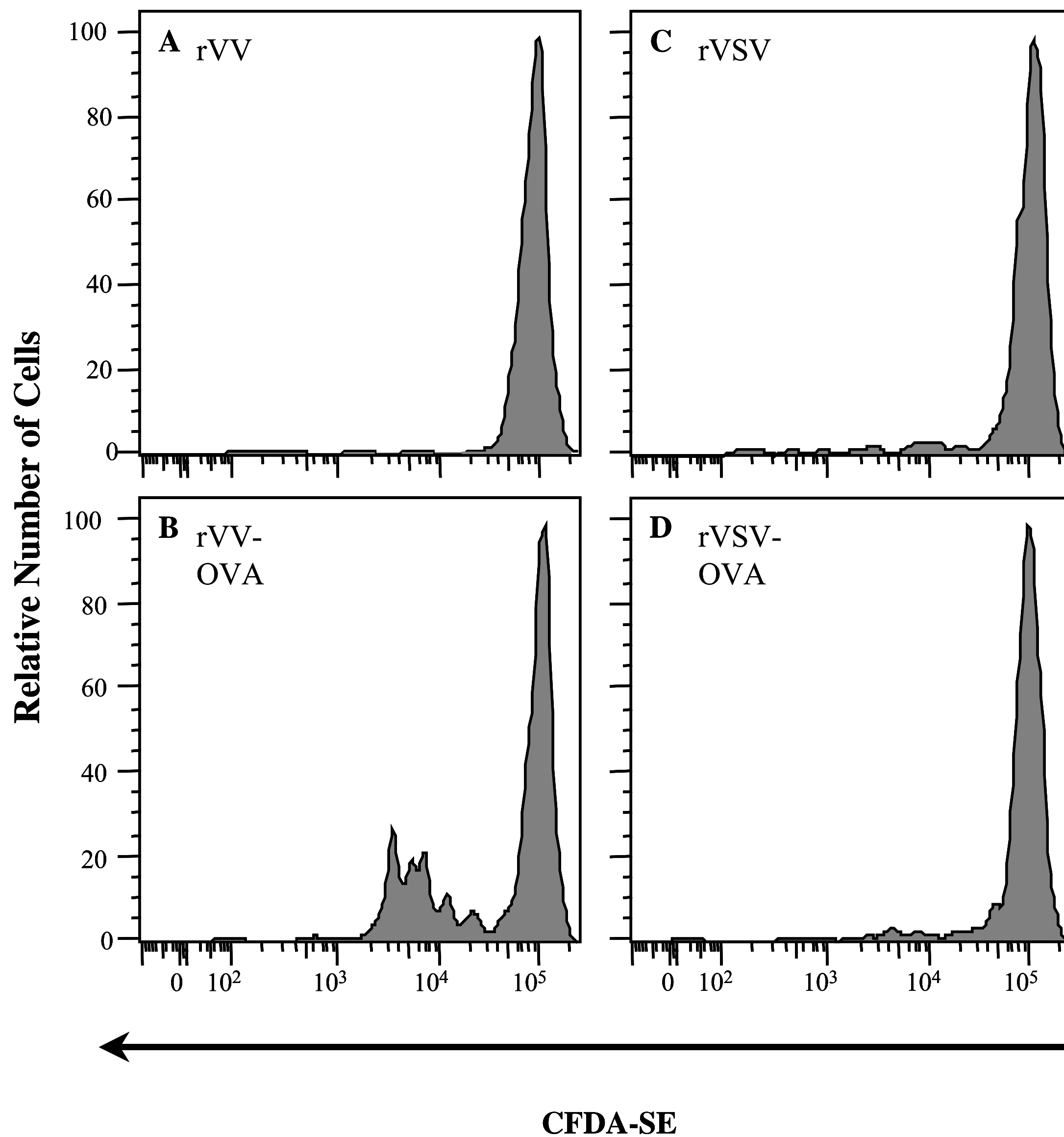

Figure 5.2 CD4+ T cell Response in TAP-/- Mice.

TAP-/- mice that had been gamma-irradiated two days prior, received an adoptive transfer of CFDA-SE-labeled OT-II.SJL cells followed by immunization with rVV (A), rVV-OVA (B), rVSV (C), or rVSV-OVA (D). Three days later CD4+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Figure is representative of 4 experiments.

controls. Mimicking the *in vivo* results, presentation of rVV-expressed OVA was TAP-independent (Figure 5.3B and F). While the TAP-dependent presentation of rVSV-expressed OVA was maintained *in vitro* (Figure 5.3H), no proliferation was observed in response to rVSV-OVA-infected wild-type BMDC (Figure 5.3D). The lack of proliferation in response to rVSV-OVA-infected wild-type BMDC indicates either that directly infected wild-type BMDC could not present rVSV-expressed OVA or that proliferation of *ex vivo* OT-II.SJL cells is not a sensitive enough system to examine the antigen processing pathways available to rVSV-expressed OVA.

T cell proliferation is not always required for acquisition of effector function (Liou et al., 1999), particularly when IL-2 is supplied in the culture medium *in vitro* (Chesler and Reiss, 2002; Huang et al., 1993; Karupiah et al., 1993; Kundig et al., 1993b). Therefore, we next tried Intracellular Cytokine Staining (ICS) to determine whether cultured OT-II.SJL cells produced cytokines in response to rVV-OVA- or rVSV-OVA-infected wild type or TAP-/- BMDC. Cultured OT-II.SJL cells must be stimulated to divide in culture; thus they can not be considered naïve T cells. We chose to examine the ability of the infected cells to stimulate interferon-γ (IFN-γ) production as IFN-γ is produced *in vivo* in response to both VV and VSV infection (Cella et al., 1997; Inaba et al., 2000; Pierre et al., 1997). Wild type and TAP-/- BMDC were infected as described above and then incubated with OT-II.SJL cells for 12 hours before performing an IFN-γ ICS. Curiously a greater percentage of IFN-γ producing OT-II.SJL cells was observed in response to rVV-OVA infected TAP-/- BMDC than to rVV-OVA infected wild type BMDC (Figure 5.4A). This increased percentage of IFN-γ producing OT-II.SJL cells was also observed in response to peptide pulsed TAP-/- BMDC and was observed in each of the three independent experiments. However, no IFN-γ producing OT-II.SJL cells above the background response to uninfected, unpulsed cells were observed in response to wild type or TAP-/- BMDC infected with rVSV-OVA (Figure 5.4A), as was seen previously in Figure 5.3. This lack of response could be

Figure 5.3

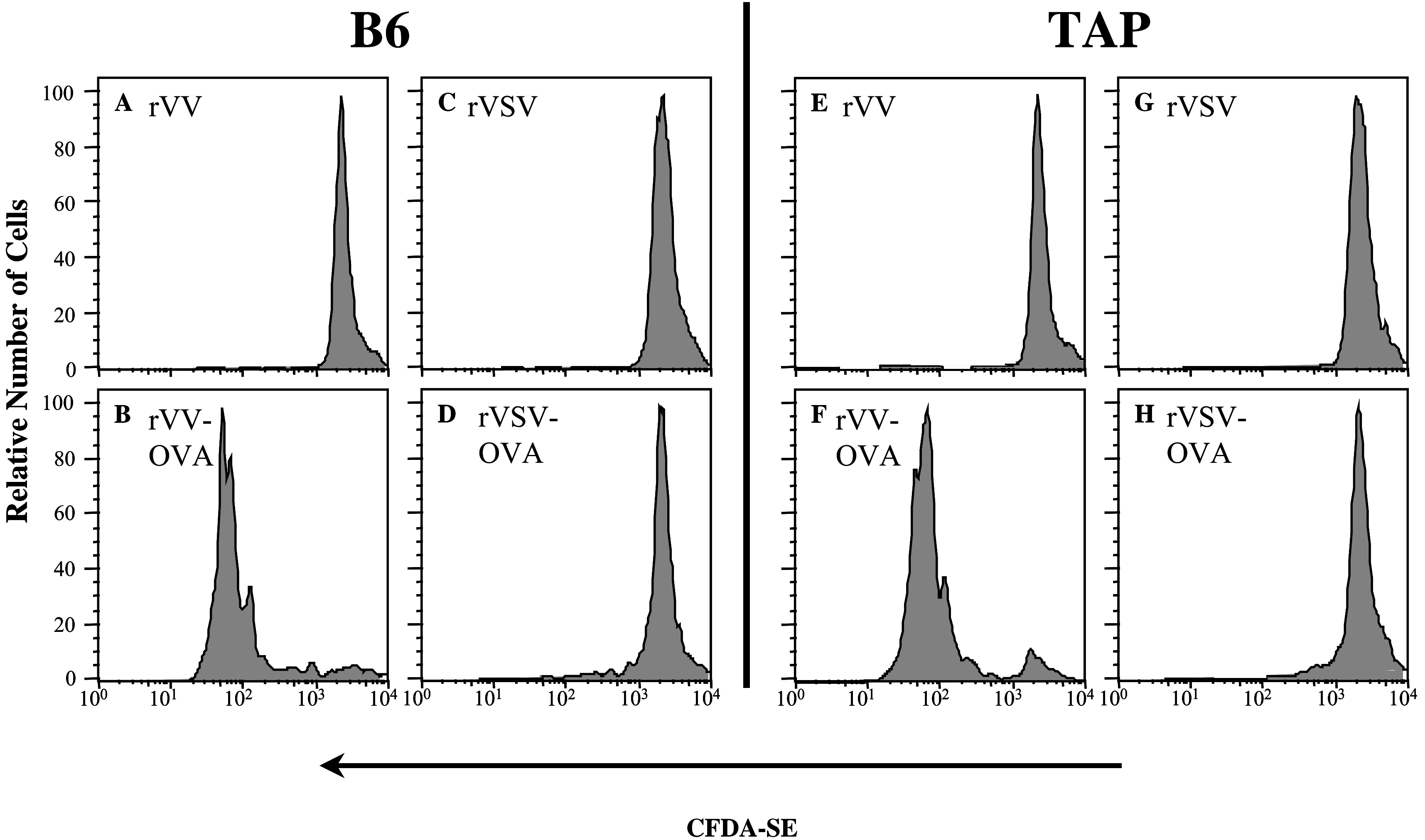

Figure 5.3 CD4+ T cell proliferation response to rVV and rVSV expressed OVA *in vitro*.

B6 (A-D) or TAP-/- (E-H) bulk BMDC were infected with rVV (A, E), rVV-OVA (B, F), rVSV (C, G), or rVSV-OVA (D, H) at an MOI=10 for 5 hours and then used to stimulate freshly isolated, CFDA-SE labeled OT-II.SJL cells. Cells were co-cultured for 96 hours before CD4+, CD45.1+ cells were evaluated for proliferation by dilution of CFDA-SE fluorescence. Each panel is representative of 4 experiments.

due to the immature nature of the dendritic cell (Ciavarra et al., 1997; Ciavarra and Burgess, 1988). It was conceivable that stimulation of a CD4+ T cell response may require mature BMDC. To address this, we repeated the IFN-γ ICS using BMDC that were matured overnight with LPS prior to infection (Figure 5.4B). IFN-γ producing OT-II.SJL cells were once again observed in response to rVV-OVA infected BMDC, but no IFN-γ producing OT-II.SJL cells above the background response were observed in response to rVSV-OVA infected wild type or TAP-/- BMDC, despite LPS maturation (Figure 5.4B). Two prior studies indicated B cells and splenic red pulp macrophages were more efficient than dendritic cells at priming T cells specific for VSV (Jenkins et al., 2001; Smith, 1984). Consequently, we infected *ex vivo* splenocytes from wild type or TAP-/- mice with the viruses and examine their ability to stimulate OT-II.SJL cell to produce IFN-γ. Infected splenocytes were unable to stimulate IFN-γ production in OT-II.SJL cells (Figure 5.4C). This result could be due to an inability of the viruses to infect splenocytes as later experiments demonstrated that only approximately 5% of *ex vivo* splenocytes were infected by rVV while less than 1% of *ex vivo* splenocytes were infected by rVSV (Figure 5.8). However, the $OVA_{323-339}$ peptide pulsed splenocytes were able to stimulate IFN-γ production, albeit at approximately half the percentage as seen in response to $OVA_{323-339}$ pulsed BMDC (Figure 5.4), indicating that the cultured T cells used in this experiment were capable of responding to antigen. The lack of response to rVSV-OVA may not be due to a lack of presentation but rather to the cytotoxicity of VSV. To address this concern, we incubated wild type BMDC that had been infected with rVSV or rVSV-OVA with neutralizing antibodies prior to co-incubation with OT-II.SJL cells. Mock antibody treated infected BMDC were included as a negative control. Despite the addition of neutralizing antibody to prevent cell lysis, no IFN-γ producing cells were observed in response to rVSV-OVA infected cells (Figure 5.5).

The inability to detect IFN-γ production in response to rVSV-OVA infected cells led us

Figure 5.4

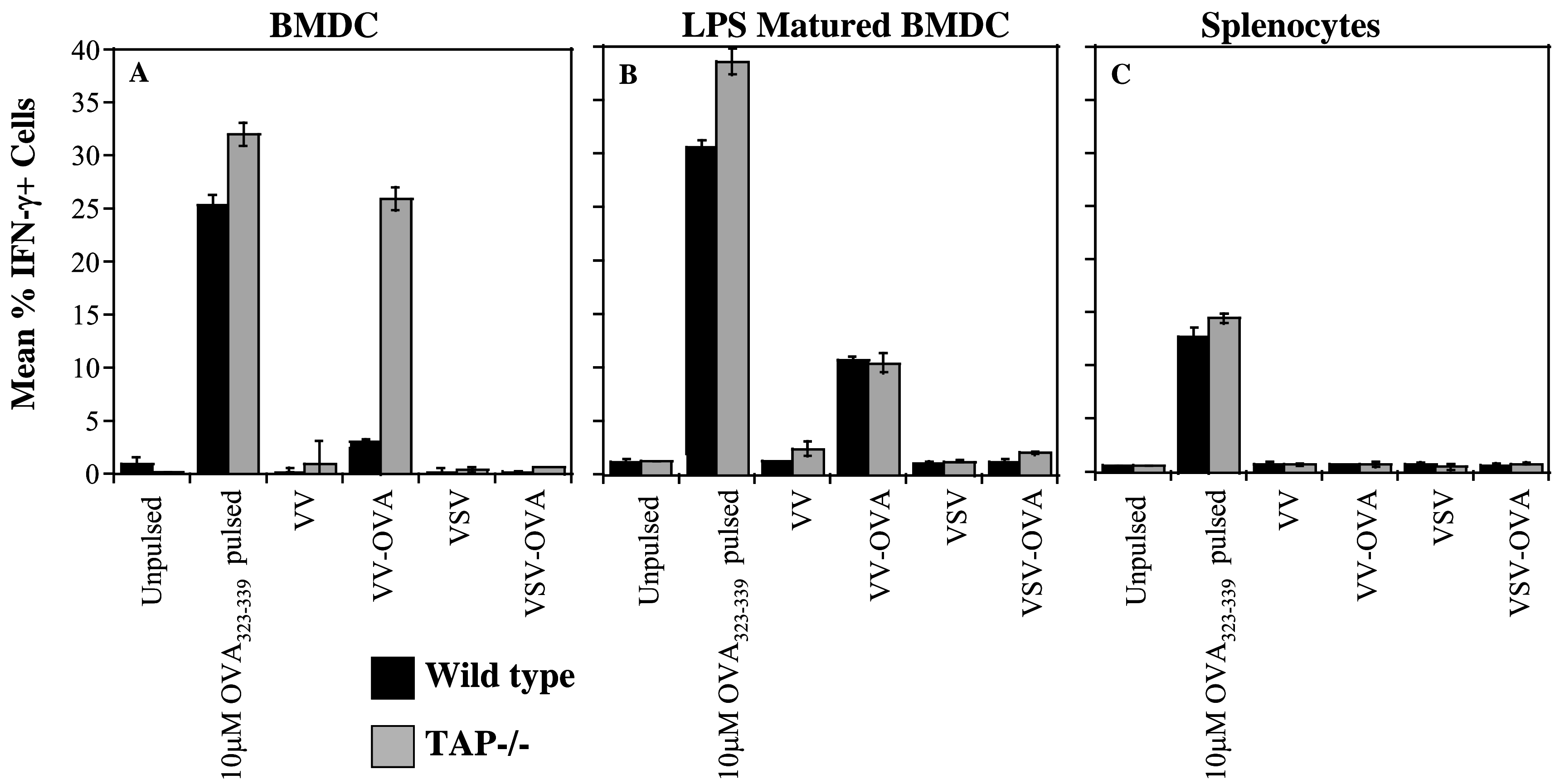

Figure 5.4 CD4+ T cell IFN-γ response to rVV and rVSV expressed OVA *in vitro*.

Wild type (black bars) or TAP-/- (gray bars) cells were infected as indicated for 30 minutes and then used to stimulate cultured OT-II.SJL cells. Cells were co-cultured for 12 hours prior to and after the addition of BFA. CD4+, CD45.1+ cells were then evaluated for their ability to produce IFN-γ. (A) BMDC, (B) BMDC stimulated with LPS overnight prior to infection, (C) Splenocytes. Each panel represents 2-3 independent experiments; errors bars show ±SEM.

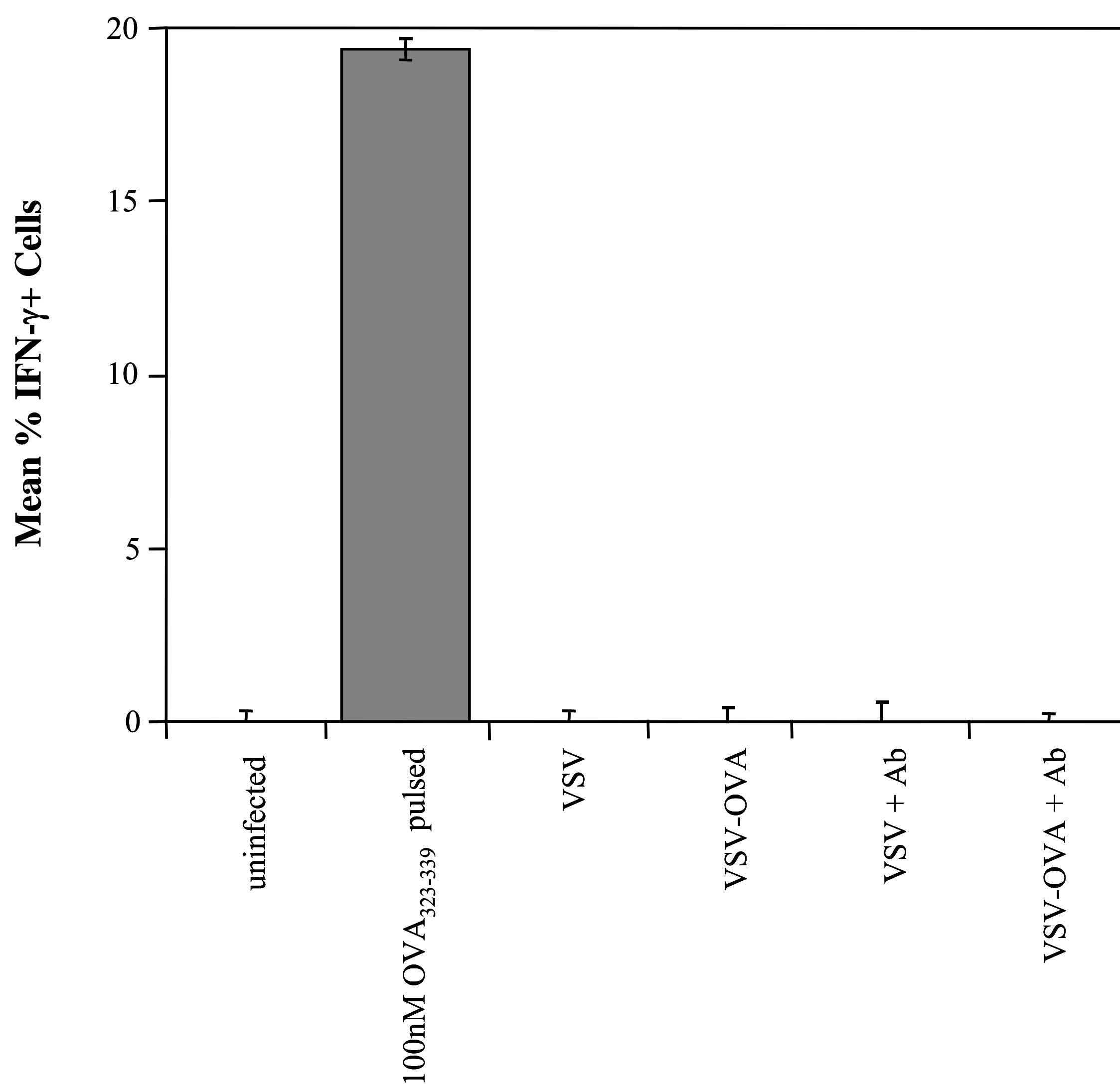

Figure 5.5 Addition of neutralizing antibody to prevent cell lysis does not enhance the response to rVSV-OVA infected BMDC.

Wild type BMDC were infected with an MOI=10 with rVSV or rVSV-OVA for 4 hours. Infected cells were then mock treated or treated with neutralizing antibody for 30 minutes prior to co-culture with cultured OT-II.SJL cells. Cells were co-cultured for 12 hours prior to and after the addition of BFA. CD4+, CD45.1+ cells were then evaluated for their ability to produce IFN-γ. Panel represents 3 independent experiments; errors bars show ±SEM.

to consider other cytokines produced by activated CD4+ T cells. Activated CD4+ T cells also produce large quantities of IL-2 (Tewari et al., 2005). Thus, we can evaluate the ability of infected BMDC to activate OT-II.SJL cells or the $OVA_{323-339}$-specific hybridoma, BO-80.10, to produce IL-2. Wild type and TAP-/- BMDC were infected as above and co-cultured with either cultured OT-II.SJL cells or BO-80.10 cells for 24 hours before supernatant was collected and IL-2 production quantitated by ELISA. Once again, OT-II.SJL cells produced IL-2 in response to both rVV-OVA infected wild type and TAP-/- BMDC (Figure 5.6A). OT-II.SJL cells did not produce IL-2 above the background levels in response to rVSV-OVA infected BMDC (Figure 5.6A). As indicated by the large error bars in Figure 5.6A, there was a great deal of variation in our results. The observed variation was consistent across three independent experiments. As both the T cells and the BMDC were cultured *ex vivo* cells, the age of the animals used to harvest the cells from, changes in the culture media (particularly the GM-CSF supernatant added to our BMDC culture media), and variability in the culture conditions could all result in the observed variability. BO-80.10 cells were not activated to produce IL-2 above background levels by any BMDC infected with rVV-OVA or rVSV-OVA (Figure 5.6B). However, BO-80.10 cells were activated to produce IL-2 in response to $OVA_{323-339}$-pulsed or rVV infected BMDC (Figure 5.6B). The activation of BO-80.10 cells by rVV-infected BMDC was seen in each of the 5 independent experiments performed, despite the fact that negative controls were moved to a separate plate after the first observation. The activation by rVV-infected BMDC is inexplicable in a cell line that is supposed to be specific for a peptide not present in rVV.

The above results suggest that infected APC do not present rVSV-expressed OVA. This may indicate that *in vivo* wild-type APC may acquire rVSV-expressed OVA from other infected cells. In an attempt to recreate this scenario, wild-type (WT3 cells) or TAP-/- fibroblasts (1E12 cells) were infected with rVV-OVA or rVSV-OVA prior to treatment with neutralizing antibodies

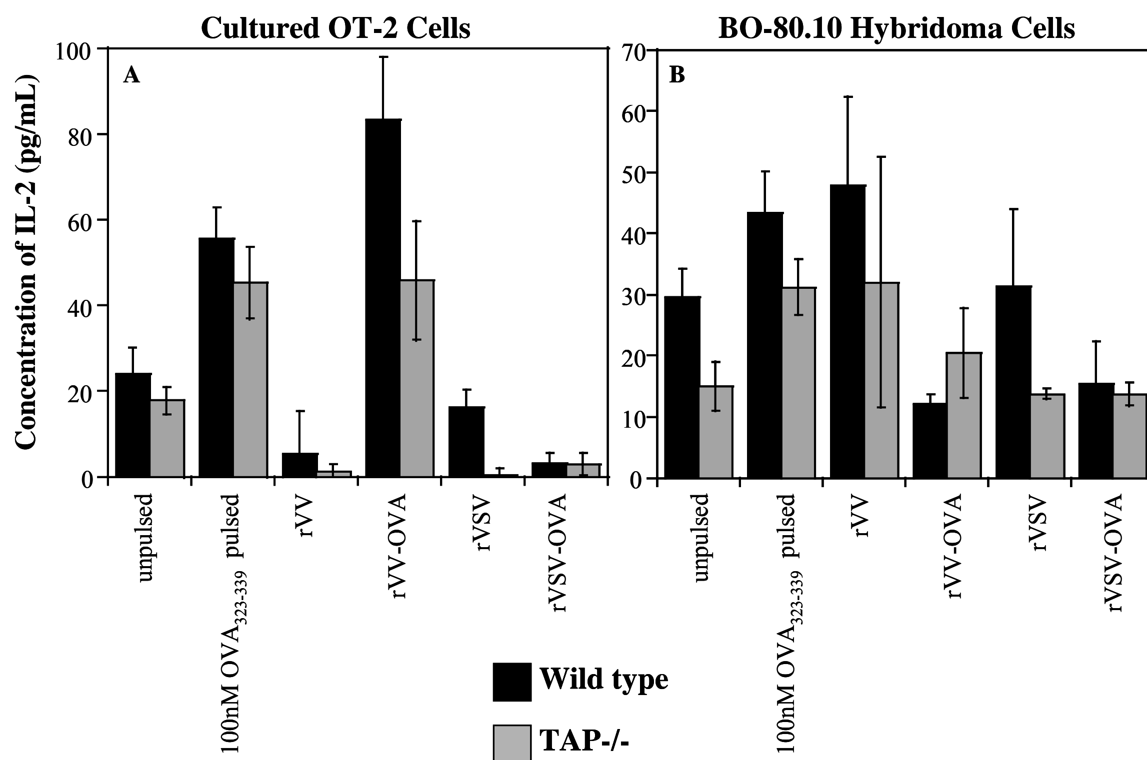

Figure 5.6 CD4+ T cell IL-2 response to rVV and rVSV infected BMDC *in vitro*.

Wild type (black bars) and TAP-/- (gray bars) BMDC were infected with an MOI=10 of rVV, rVV-OVA, rVSV, or rVSV-OVA for four hours. rVSV infected cells were then treated with neutralizing antibody for 30 minutes prior to co-culture with cultured OT-II.SJL cells (A) or BO-80.10 hybridoma cells (B). Cells were co-cultured for 24 hours before the amount of IL-2 produced was quantitated by ELISA. Panel A represents 3 independent experiments while panel B represents 5 independent experiments; errors bars show ±SEM.

and co-incubation with wild-type or TAP-/- BMDC. Cultured OT-II.SJL cells were then added and co-incubated for 12 hours before an IFN-γ ICS was performed. Similar percents of IFN-γ producing OT-II.SJL cells were stimulated by rVV-OVA, regardless of the presence of TAP in either the fibroblasts or BMDC (Figure 5.7). The percent of IFN-γ producing OT-II.SJL cells stimulated by rVV-OVA was approximately one-third of the percent of IFN-γ producing OT-II.SJL cells stimulated by rVSV-OVA (Figure 5.7). This may be due to an enhanced response stimulated by rVSV expressed OVA, but it is likely due to the fact that the majority of OVA expressed by rVV is sequestered within the cell (Figure 4.3A) unlike OVA expressed by rVSV, which is secreted from the infected cell (Figure 4.3B). The differing level of response could also be due to the different expression level of OVA by the two viruses (Figure 4.3). Once the error bars are accounted for, TAP-/- fibroblasts infected with rVSV-OVA and incubated with wild type BMDC (5.7A) stimulated roughly the same number of IFN-γ producing OT-II.SJL cells as TAP-/- fibroblasts infected with rVSV-OVA and incubated with TAP-/- BMDC (Figure 5.7B). These results suggest that APC can present rVSV-expressed OVA acquired from rVSV-OVA infected cells on MHC class II, regardless of the presence of TAP. However, this result is in contrast to our TAP-dependent MHC class II presentation of rVSV-expressed OVA *in vivo*.

5.2.3 Determining which antigen presenting cells are infected with rVV and rVSV

One concern not addressed in the above *in vitro* experiments was the ability of rVV and rVSV to both infect the APC and express OVA. Previous experiments (Figure 4.3) demonstrated that the viruses could infect and produce OVA in fibroblasts, explaining the results in response to rVSV-OVA in Figure 5.7. After concluding our *in vitro* experiments, we were able to obtain an rVSV that expresses the green fluorescent protein (GFP) under the same promoter used to express OVA (we already possessed a similar rVV). Thus, we examined the ability of these viruses to

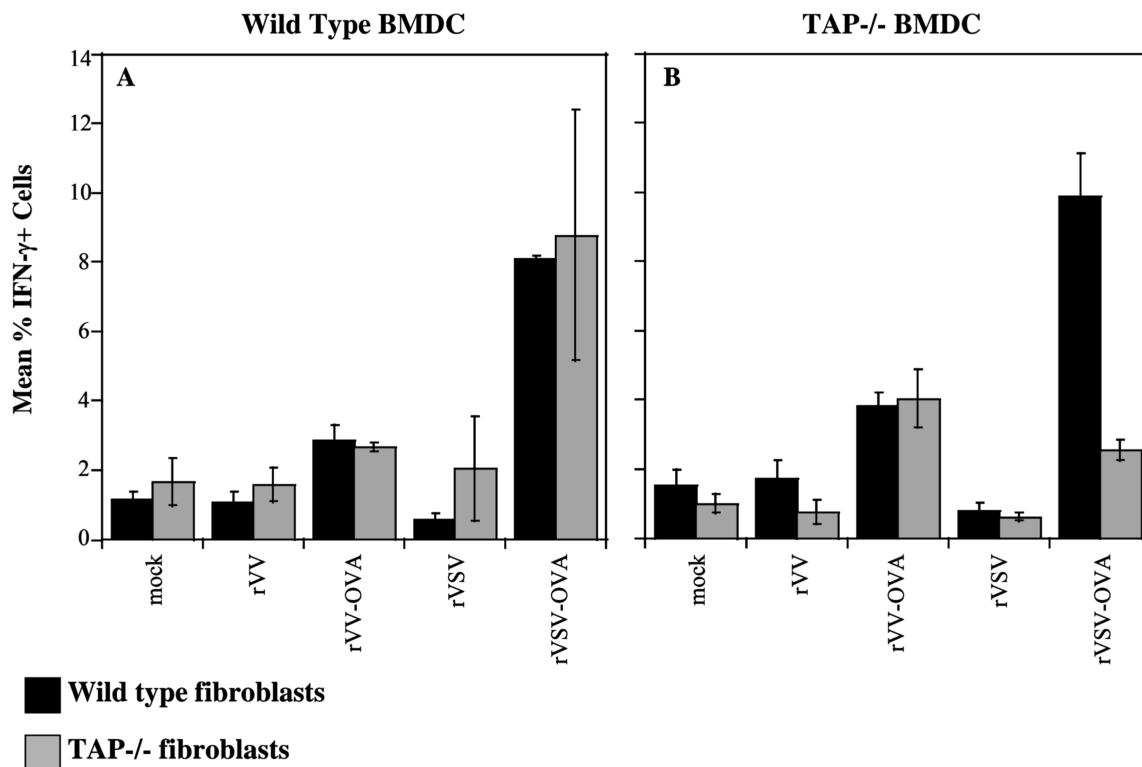

Figure 5.7 TAP is not required in the antigen donor cell for the presentation of rVSV-expressed OVA *in vitro*, but the presence of TAP greatly enhances presentation.

Wild type (black bars) and TAP-/- (gray bars) fibroblasts were infected with an MOI=10 of rVV, rVV-OVA, rVSV, or rVSV-OVA for 4 hours. rVSV infected cells were then treated with neutralizing antibody for 30 minutes prior to co-culture with B6 (A) or TAP-/- (B) BMDC. Fibroblasts and BMDC were incubated together for 1 hour prior to the addition of cultured OT-II.SJL cells. Cells were co-cultured for 12 hours prior to and after the addition of BFA. CD4+, CD45.1+ cells were then evaluated for their ability to produce IFN-γ. Panels represent 3 independent experiments; errors bars show ±SEM.

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infect the antigen presenting cells and express GFP. BMDC and splenocytes, the two types of antigen presenting cells utilized in our *in vitro* experiments were infected with our GFP viruses and evaluated for GFP expression by flow cytometry. The processing of spleens can chemically or mechanically shear receptors from the cell surface of splenocytes, potentially preventing the viruses from entering the cell. Thus, two different methods of processing the spleens, ACK lysis and collagenase D digestion, were used. BHK cells, fibroblasts known to be permissive for both rVV and rVSV infection, were used as positive controls. A large percentage of BHK cells expressed GFP in response to infection by rVV (92.3%) and rVSV (81%) encoding GFP. BMDC were also infected by both rVV and rVSV, with 32.4% and 10.4% of cells expressing GFP respectively (Figure 5.8). This indicates that the BMDC used in our *in vitro* experiments were likely infected and expressing OVA. A low percentage of ACK (5.45%) and collagenase (5.9%) prepared splenocytes expressed GFP after infection with an rVV expressing GFP. No GFP expression was seen in either splenocyte preparation infected with the rVSV expressing GFP (Figure 5.8). This does not indicate that these cells are not infected, only that they do not express GFP. However, these results may explain why we did not see a response to rVV-OVA or rVSV-OVA infection of wild type splenocytes in Figure 5.4.

5.2.4 Is TAP required for MHC class II presentation of VSV epitopes ?

Studies with influenza demonstrated that presentation of MHC class II epitopes present within the same protein can have different requirements for TAP (Burkhart et al., 1994a). Thus, we examined whether the presentation of an MHC class II epitope present in the VSV glycoprotein, p41 (Buller et al., 1987; Cassell and Forman, 1988; Fernando et al., 2002; Husmann and Bevan, 1988; Keene and Forman, 1982; Rahemtulla et al., 1991; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004; Wu and Liu, 1994), was dependent on TAP *in vivo*. Wild type

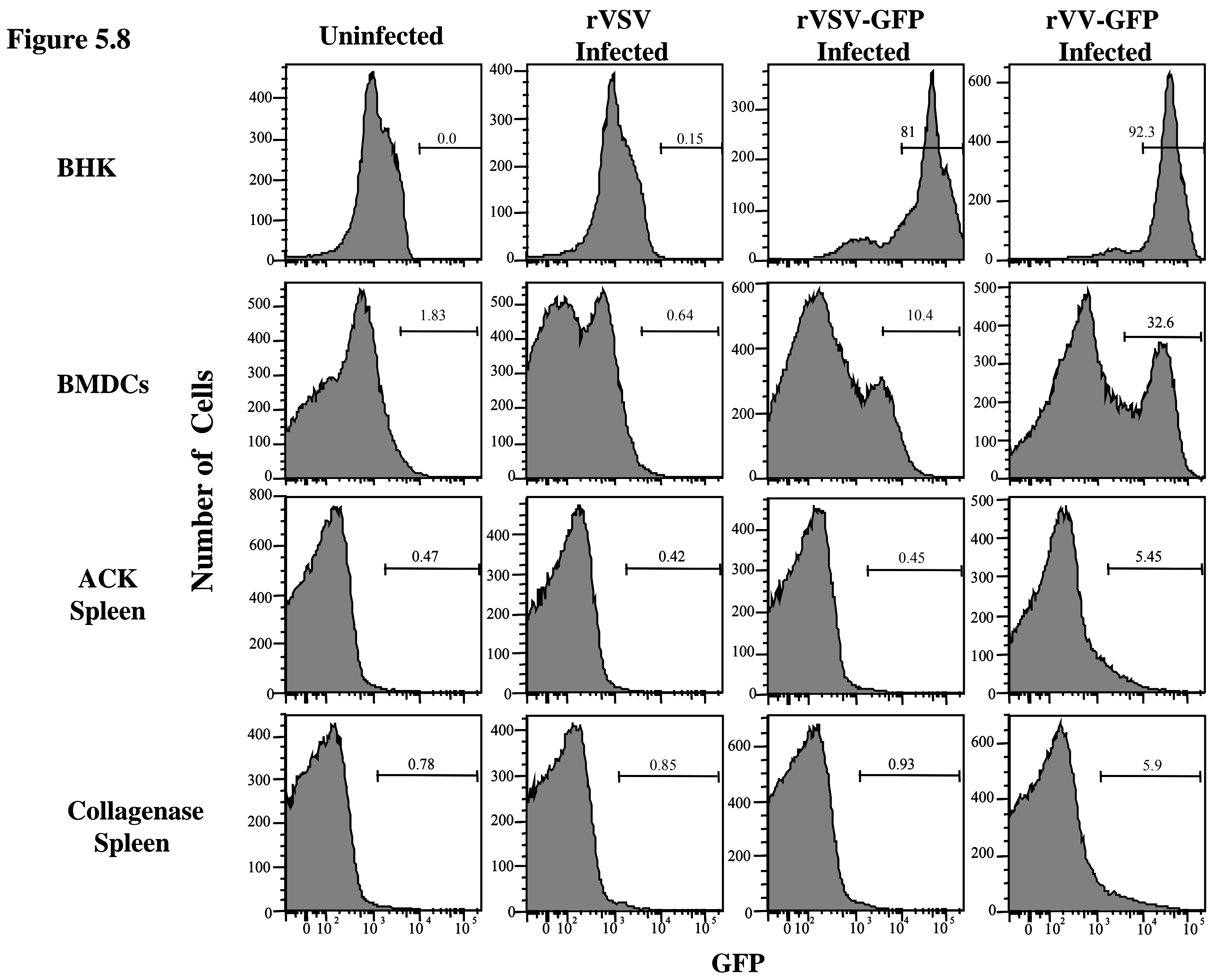
Figure 5.8
Uninfected
rVSV Infected
rVSV-GFP Infected
rVV-GFP Infected
BHK
BMDCs
ACK Spleen
Collagenase Spleen
Number of Cells
GFP
0.0
0.15
81
92.3
1.83
0.64
10.4
32.6
0.47
0.42
0.45
5.45
0.78
0.85
0.93
5.9

Figure 5.8 Which cell types are infected by rVV and rVSV?

Fibroblasts (BHK), cultured BMDC (BMDC), splenocytes isolated by ACK lysis (ACK spleen) and splenocytes isolated by collagenase D treatment (collagenase spleen) were infected as indicated. Four hours after infection, cells were evaluated by flow cytometry for expression of GFP. Uninfected cells and rVSV infected cells were included as negative controls. Gates were determined based on the rVSV infected cells. Figure is representative of 5 experiments.

(B6) and TAP-/- mice were infected with rVSV or rVSV-OVA. Six days later, the percent of CD4+ T cells in the spleen that produced IL-2 in response to stimulation with BMDC pulsed with either the p41 determinant or $OVA_{323-339}$ was determined by ICS. All rVSV or rVSV-OVA infected mice had CD4+ T cells that produced IL-2 in response to BMDC pulsed with the VSV glycoprotein determinant p41 (Figure 5.9). It should be noted that this is the first example of an *ex vivo* CD4+ T cell ICS for VSV. Surprisingly, rVSV-OVA infected wild type and TAP-/- mice both had CD4+ T cells that produced IL-2 in response to BMDC pulsed with the OVA determinant $OVA_{323-339}$ (Figure 5.9). This is contrary to the previous *in vivo* results observed in Figure 5.2. The major difference between the experiments presented in Figures 5.2 and 5.9 is that the TAP-/- mice were not irradiated for the experiments in Figure 5.9 because we were examining the naïve CD4+ T cell response to both VSV and OVA epitopes in TAP-/- mice rather than adoptively transferring CD4+ T cells specific for $OVA_{323-339}$.

5.3 Discussion

Few studies have examined the ability of recombinant viral vectors to stimulate CD4+ T cell immunity to a transgene, despite demonstration that CD4+ T cells are important in the establishment of CD8+ T cell memory (Carmichael et al., 1996; Malnati et al., 1992; Tewari et al., 2005). Thus, in this study we sought to compare the MHC class II processing and presentation pathways available to OVA when it is expressed by either rVV or rVSV. As mentioned in the introduction, TAP-dependent MHC class II presentation was observed while running controls for *in vivo* experiments presented in Chapter 4. Therefore, our study began by characterizing the antigen processing and presentation pathways available to virally expressed OVA in wild type mice. OVA expressed by both rVV and rVSV stimulated proliferation in OVA-specific transgenic CD4+ T cells in wild-type (B6) mice (Figure 5.1). The level of proliferation

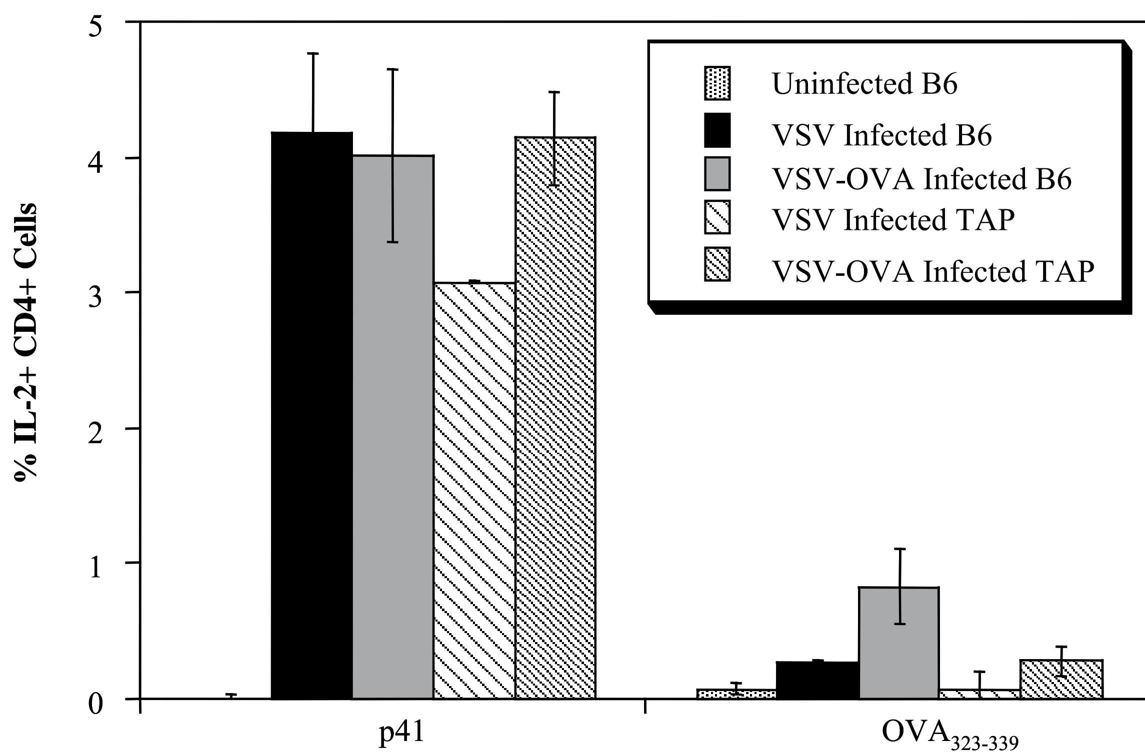

Figure 5.9 CD4+ T cell Responses are generated to both the MHC Class II VSV determinant as well as to the MHC Class II OVA determinant in TAP-/- mice.

B6 or TAP-/- mice were either unimmunized or immunized with $5x10^6$PFU/mouse with either rVSV or rVSV-OVA as indicated. Six days later, the percent of responding CD4+ T cells present in spleens were determined by their expression of IL-2 in response to BMDC pulsed with either the MHC class II VSV glycoprotein determinant (p41) or the MHC class II OVA determinant ($OVA_{323-339}$). CD4+ cells were evaluated for their ability to produce IL-2 by ICS. Results are representative of 3 experiments; errors bars show ±SEM.

stimulated was similar for both rVV and rVSV expressed OVA, despite rVV expressed OVA being sequestered within the infected cell (Figure 4.3). MHC class II presentation typically involves endosomal processing of exogenous antigen; hence it was uncertain whether the sequestration of rVV-expressed OVA would affect its ability to access the MHC class II processing pathway. Next, we further examined the ability of rVV-OVA and rVSV-OVA to stimulate OVA-specific CD4+ transgenic T cells in TAP-/- mice. Only rVV-expressed OVA was capable of stimulating proliferation in OVA-specific CD4+ transgenic T cells in TAP-/- mice (Figure 5.2), suggesting that TAP is involved in both the MHC class I and MHC class II processing pathways for rVSV-expressed OVA.

Previous examples of TAP-dependent MHC class II presentation involved the infected cell presenting the endogenously expressed antigen (Carmichael et al., 1996; Malnati et al., 1992; Tewari et al., 2005). To examine whether the infected cell was also presenting OVA, we moved to an *in vitro* system. Infected BMDC, LPS-matured BMDC and splenocytes were not able to present rVSV-expressed OVA on MHC class II regardless of the presence of TAP (Figures 5.3, 5.4, 5.5 and 5.6). Infected splenocytes were also unable to present rVV-expressed OVA (Figure 5.4C). The lack of OVA presentation in splenocytes is likely due to an inability of splenocytes to be infected or express OVA as experiments with rVV- and rVSV-GFP (where GFP was under the same promoter as OVA) revealed that less than 6% of splenocytes expressed GFP after rVV-GFP infection and no splenocytes expressed GFP after rVSV-GFP infection (Figure 5.8). However, infected BMDC and LPS-matured BMDC were able to present rVV-expressed OVA regardless of the presence of TAP (Figures 5.3 and 5.4). The lack of presentation of rVSV-expressed OVA was not due to an inability of rVSV to infect BMDC or an inability of rVSV to express OVA as 10.4% of BMDC infected with rVSV-GFP expressed GFP (where GFP was under the same promoter as OVA) (Figure 5.8). These experiments, together with the knowledge that rVSV-expressed OVA is secreted from infected cells (Figure 4.3) suggest that rVSV-expressed OVA is

not presented by infected BMDC. This differs from the previously reported examples of TAP-dependent MHC class II presentation which involved presentation of endogenously expressed antigens (Ciavarra et al., 1997; Ciavarra and Burgess, 1988). Thus, we examined whether MHC class II presentation of OVA secreted from infected cells was TAP-dependent. OVA expressed by both rVV and rVSV was able to stimulate cultured OVA-specific transgenic CD4+ T cells to produce IFN-γ regardless of the presence of TAP in either the infected fibroblasts or the BMDC (Figure 5.7). This result suggests that rVV-expressed OVA can be presented by the infected BMDC or secreted and presented by an uninfected antigen presenting cell. Although the Western blot in Figure 4.3 suggests that OVA expressed by rVV is sequestered within the infected cell, it is possible that an antigenically relevant amount of OVA is secreted from the cell and is available for MHC class II processing and presentation by uninfected antigen presenting cells. It is also possible that rVV-expressed OVA is secreted from infected BMDC as the western blots only examined the secretion of OVA from infected fibroblasts (Figure 4.3). The ability of OVA expressed from rVSV to stimulate cultured OVA-specific transgenic CD4+ T cells to produce IFN-γ regardless of the presence of TAP in either the infected fibroblasts or the BMDC (Figure 5.7) suggests that rVSV-expressed OVA is only presented by an uninfected antigen presenting cells. However, based on the results in Figure 5.9, at least 10% of BMDC were infected by rVSV and expressed GFP. This raises the possibility that although BMDC are infected by rVSV (Figure 5.9), infected BMDC might not secrete rVSV-expressed OVA as the secretion of OVA was only examined in infected fibroblasts (Figure 4.3). The 5-fold greater response seen to rVSV-expressed OVA than to rVV-expressed OVA (Figure 5.7) may be explained by the fact that OVA expressed by rVSV is more readily secreted than that expressed by rVV (Figure 4.3), although as noted above, the secretion of OVA was only examined in fibroblasts (Figure 4.3). The results of Figure 5.7, together with those of Figures 5.3-5.6, indicate that rVSV-expressed OVA is not presented on MHC class II by infected BMDC but is secreted from an infected cell

and presented by an uninfected BMDC in a TAP-independent manner. This result is contrary to our *in vivo* result where presentation of rVSV-expressed OVA was TAP-dependent.

As demonstrated by the error bars in Figure 5.4-5.7, our *in vitro* results were highly variable. This variability was seen in each of the independent experiments performed *in vitro*. The most likely culprit for the variability seen in our *in vitro* assays was the differences in the ages of the animals used to harvest the cells for growing BMDC. During the time period when these assays were run, our lab was having difficulties breeding TAP-/- mice. Consequently, almost all of the TAP-/- mice used to harvest bone marrow were no longer of breeding age (older than 6 months). When possible, the wild-type B6 mice were age-matched with the TAP-/- mice when setting up BMDC cultures. Consequently, the data used to generate the panels depicted in Figure 5.4-5.7 were from experiments where the age of the mice as sources of bone marrow for BMDC cultures were most closely matched so that age differences would not confound interpretation of the results. An additional factor in the variability of the BMDC cultures was the fact that we used supernatant from a GM-CSF-producing hybridoma as our source of GM-CSF in the BMDC media. While care was taken so that the wild type BMDC and the TAP-/- BMDC received the exact same media (made with GM-CSF from the same supernatant harvest), there were likely differences in the concentrations of GM-CSF in the BMDC media from experiment to experiment. Variability in the *in vitro* assays depicted in Figures 5.4-5.7 could also have resulted from the cultured OT-II.SJL T cells used in these experiments. While care was taken to assay the sensitivity of the cultured T cells using peptide-pulsed wild type BMDC, the cultured OT-II.SJL T cells varied in their response to the same concentration of $OVA_{323-339}$ peptide-pulsed BMDC within a single experiment (see error bars on Figures 5.4-5.6).

Despite the variations of the *in vitro* assays, one possible explanation for the discrepancies between our *in vivo* and *in vitro* results was the requirement to gamma-irradiate TAP-/- mice before performing an experiment with using antigen-specific transgenic CD4+ T

cells. Gamma-irradiation eliminates the alloresponse of the TAP-/- cells to the increased level of MHC class I expressed on the surface of the adoptively transferred cells. However, gamma-irradiation also eliminates cells like B cells and splenic red pulp macrophages- cell types reported (although much discounted) to be more efficient at activating T cells in VSV infection (Tewari et al., 2005). Previous studies had also indicated that presentation of MHC class II epitopes present within the same influenza protein can have different requirements for TAP (Ciavarra et al., 1997; Ciavarra and Burgess, 1988). Thus, two possible explanations existed for the discrepancies between our *in vivo* and *in vitro* results. To address these discrepancies, the ability of CD4+ T cells to produce IL-2 in response to OVA or the VSV glycoprotein (which is expressed under the same promoter as OVA in rVSV) was assessed *ex vivo* following infection of wild type and TAP-/- mice with rVSV and rVSV-OVA. All rVSV or rVSV-OVA infected mice had CD4+ T cells that produced IL-2 in response to BMDC pulsed with the VSV glycoprotein determinant (Figure 5.9). Contrasting to the *in vivo* experiments in Figure 5.2, rVSV-OVA infected wild type and TAP-/- mice both had CD4+ T cells that produced IL-2 in response to BMDC pulsed with the MHC class II OVA determinant (Figure 5.9). This result suggests that rVSV-expressed OVA is not dependent on TAP for presentation on MHC class II but is instead dependent on specific populations of antigen presenting cells for the activation of T cells. Our results in Figure 5.2 likely indicate that the type of antigen presenting cell needed to activate T cells was not available due to gamma-irradiation. Furthermore, this result agrees with the previously published findings of that B cells and splenic red pulp macrophages are likely to be involved in the activation of T cells in VSV infection (Ciavarra et al., 2000). An additional study from the Ciavarra group demonstrated that while dendritic cells could and did present VSV-epitopes, T cells were only activated when macrophages were present (Ciavarra et al., 1997; Ciavarra and Burgess, 1988; Ciavarra et al., 2000). This study, together with our findings emphasizes that the activation of T cells requires not only presentation of antigen, but the proper cells and signals to activate specific

T cells *in vivo*. It would be interesting to determine if lack of splenic red pulp macrophages and B cells also affected the activation of OVA-specific T cells in wild type mice. In other words, does rVSV expression of OVA require OVA to be presented by the same antigen presenting cells that would normally activate VSV-specific T cells?

In summary, we have demonstrated that activation of OVA-specific CD4+ T cells by rVSV-expressed OVA requires the presence of specific populations of antigen presenting cells, not the presence of TAP. We also demonstrated that rVV-expressed OVA is likely presented on MHC class II by the infected cell while rVSV-expressed OVA is likely secreted from the infected cell and presented by an antigen presenting cells. While our *in vitro* studies used BMDC as antigen presenting cells, they also used cultured OVA-specific CD4+ T cells. It is likely that any requirement for additional stimulation not provided by the BMDC was abrogated by the presence of T cell growth factors in the culture media. Further *in vitro* studies are likely to be ineffective since our experiments demonstrate that both the chemical and the mechanical isolation of splenic cells render them unable to be infected by rVSV. Our *in vivo* studies have demonstrated that the antigen presenting cells required to activate CD4+ T cells to VSV and VSV-expressed antigens are ablated by gamma-irradiation, which fits with published reports that B cells and splenic macrophages are required (Arnold et al., 1995; Binder and Srivastava, 2005; Suto and Srivastava, 1995; Udono and Srivastava, 1993) for the activation of T cells to VSV antigens. Our *in vivo* studies have also resulted in the development of a CD4+ T cell ICS for VSV; allowing for further characterization of the CD4+ T cell response to VSV. However, future studies are needed to assess whether the CD4+ T cells stimulated by rVV- and rVSV-expressed OVA are sufficient to establish a CD8+ T cell memory response, a requirement for rational vaccine design.

Chapter 6

Discussion

6.1 General Implications and Significance

The research in this thesis has focused on methods of activating T cells *in vivo*. The work presented involved two aspects of activating T cells *in vivo*. One aspect investigated the requirements for direct and cross-priming CD8+ T cells *in vivo*. The other aspect focused on the antigen presentation pathways available to a model antigen when expressed by two different viral vectors.

6.1.1 Requirements for Direct and Cross-Priming CD8+ T Cells *In Vivo*

Investigation of the requirements for direct and cross-priming revealed that the two pathways utilize different pools of antigen. Direct presentation requires newly synthesized rapidly degraded antigen while cross-priming requires long-lived stable antigen. Direct presentation is an active process for the antigen presenting cell with requirements for the protein synthesis of antigen as well as other factors for presentation to occur. However, *in vivo* cross-priming was demonstrated to have no requirement for proteasomal processing, protein synthesis or vesicular transport of the antigen or other cellular factors in the antigen donor cell. Our findings are in direct contrast to the hypothesis that chaperone-bound peptides are the form of antigen transferred in the *in vivo* cross-priming pathway (Arnold et al., 1995; Binder and Srivastava, 2005; Suto and Srivastava, 1995; Udono and Srivastava, 1993). We have demonstrated that antigen donated to the cross-priming pathway must persist within the antigen

donor cell for hours prior to antigen donation. While chaperones are known to transiently associate with unfolded proteins as part of their normal housekeeping functions (Kleizen and Braakman, 2004; Martin et al., 1991; Molinari et al., 2002; Ostermann et al., 1989), the requirements of the cross-priming pathway would dictate both promiscuous and prolonged chaperone-antigen interactions. Such interactions would be costly to the cell as they would sequester chaperones from their normal cellular functions. As our research indicates that antigen donation does not require any of the cellular processes tested, it is unlikely that the form of antigen donated would require the donor cell to commit energy to maintain that antigen until it could be donated. Our findings suggest that the cross-priming pathway evolved to allow the generation of immune responses to pathogens that were not directly presented either due to the inability to infect antigen presenting cells or due to expression of immune evasion molecules that subvert the direct priming pathway. The lack of requirement for any of the tested cellular processes to transfer antigen makes the cross-priming pathway resistant to modification and manipulation.

While our studies addressed the form of antigen required for cross-priming *in vivo* and the requirement for various cellular processes in the antigen donor cell for donation of antigen to the cross-priming pathway, the question of how antigen is transferred from the antigen donor cell to the antigen presenting cell *in vivo* has not been addressed. Previous studies have suggested that cell-associated antigens are cross-primed more efficiently than soluble antigens (Carbone and Bevan, 1990; Li et al., 2001). The data shown in Figure 4.9 support this theory as rVV-expressed ovalbumin (OVA), which was sequestered with the cell (Figure 4.3) stimulated more cross-priming than rVSV-expressed OVA, which was secreted from the cell (Figure 4.3). Together with our data that implies that cross-priming is a passive process for the antigen donor cell, this data implies that the antigen donor cell could simply be packaging that concentrates and protects an antigen for delivery to the cross-priming pathway. To address the possibility that the

antigen donor cell is merely packaging, a synthetic phospholipid vesicle containing a model antigen could be used. If the purpose of the antigen donor cell (beyond expressing the model antigen) is to package antigen for delivery to the cross-priming pathway, one would expect that antigen inside a synthetic phospholipid pathway would be sufficient to stimulate antigen-specific T cells. However, if the antigen donor cell has a function beyond serving as a package for antigen donation, then one would expect that a synthetic phospholipid vesicle containing a model antigen would be insufficient to activate antigen-specific T cells. Furthermore, by constructing synthetic phospholipid vesicles such that they resemble cell membranes of apoptotic cells, the role of apoptosis in the donation of antigen to the cross-priming pathway *in vivo* could also be addressed. The importance of the antigen donor cell and the importance of apoptosis in stimulating cross-priming could be very important to understanding how best to target antigens to the cross-priming pathway in order to enhance T cell activation.

6.1.2 Antigen Processing and Presentation Pathways Available to Virally-Expressed Antigens

Examination of the antigen presentation pathways available to a viral vector-expressed antigen revealed that the viral vector does influence the antigen presentation pathways available to the antigen. These experiments utilized two potential vaccine vectors, recombinant Vaccinia Virus (rVV) and recombinant Vesicular Stomatitis Virus (rVSV) to express ovalbumin (OVA) as the model antigen as it is a soluble protein unlikely to exert an effect on the viral vector. Western blots revealed that rVV-OVA expressed one thousand-fold more OVA than rVSV-OVA (Figure 4.3). OVA is normally a soluble, secreted protein, yet Western blots also determined that rVV-expressed OVA was sequestered within the fibroblast while rVSV-expressed OVA was secreted from the cell (Figure 4.3). The probable reason for the sequestration of OVA with the rVV-

infected cell is that rVV infection induces reorganization of lipid-bilayers within the cell, likely resulting in an inability to secrete OVA (Dales and Mosbach, 1968; Risco et al., 2002; Sodeik et al., 1993; Sodeik and Krijnse-Locker, 2002). In our examination of the MHC class I processing pathways available to rVV- and rVSV expressed OVA, we discovered that rVV-infection permits OVA to access the TAP-independent cross-priming pathway. This finding suggests that rVV expression should enhance presentation of OVA to CD8+ T cells, yet rVSV expression of OVA stimulated a 5-fold greater OVA-specific CD8+ T cell response in wild type mice, likely due to direct priming of rVSV-expressed OVA. The greater total OVA-specific CD8+ T cell response to rVSV-expressed OVA in the absence of access to the TAP-independent cross-priming pathway raise the question of how important the TAP-independent cross-priming pathway is for activating antigen-specific CD8+ T cells. In particular, it raises the question of whether there are functional differences in the types of CD8+ T cells stimulated by the TAP-independent cross-priming pathway. Immunizing mice with either rVV-OVA or rVSV-OVA, waiting 6 days and then performing functionally analyzing the OVA-specific CD8+ T cells, could answer these questions. Additionally, the importance of the CD8+ T cells stimulated by the TAP-independent cross-priming pathway in establishing CD8+ T cell memory would also need to be examined to aid in the design of vaccines. Ideally, multiple model antigens would need to be examined in order to understand the importance of the TAP-independent cross-priming pathway in activating antigen-specific CD8+ T cell memory.

The finding that rVV-expression, but not rVSV-expression, permits OVA to access the TAP-independent pathway should also be re-examined in non-irradiated TAP knock out mice. Our findings from our Chapter 5 experiments indicate specific antigen presenting cell populations not present in irradiated mice are required to present rVSV-expressed OVA to CD4+ T cells. Thus, the possibility that rVSV may require a specific antigen presenting cell population to permit OVA to access the TAP-independent cross-priming pathway should be examined.

The inability of rVSV-OVA-infected BMDC to activate OVA-specific CD4+ T cells *in vitro* is peculiar when considered with our other results. First, based on our results in Figure 5.8, at least 10% of BMDC were infected with rVSV and expressed GFP (GFP is expressed on the same transcript as OVA is by the rVSV-OVA virus). Despite only 10% of BMDC being infected and expressing the transgene, wild type rVSV-OVA-infected BMDC stimulate OVA-specific CD8+ T cells (Figure 4.6). Yet, no OVA-specific CD4+ T cells are activated by rVSV-OVA-infected BMDC (Figures 5.3-5.6). Several factors could explain the inability of rVSV-OVA infected wild type BMDC to activate OVA-specific CD4+ T cells. Fibroblasts infected with rVSV-OVA secreted OVA into the supernatant (Figure 4.3). However, the secretion of rVV- and rVSV-expressed OVA was never examined for BMDC. Lack of secretion of rVSV-expressed OVA from BMDC could explain why BMDC don't present OVA on MHC class II. As reported in Chapter 5, rVSV-OVA-infected BMDC could present OVA on MHC class II, but could be unable to activate the CD4+ T cells (Ciavarra et al., 2000). In this case, it would be necessary to stain rVSV-OVA-infected BMDC with an antibody specific for MHC class II-OVA$_{323-339}$ to demonstrate that BMDC are capable of presenting rVSV-expressed OVA on MHC class II. Alternatively, B cells or splenic red pulp macrophages could be used as antigen presenting cells in an *in vitro* assay as these cells have been demonstrated to present VSV antigens and activate VSV-specific T cells (Ciavarra et al., 1997; Ciavarra and Burgess, 1988; Ciavarra et al., 2000). The last possibility that could explain why no OVA-specific CD4+ T cell response was seen *in vitro* to rVSV-OVA-infected BMDC involves the culture of the BMDC. BMDC were made from cultures of bone marrow harvested from wild type or TAP-/- mice. Variations in the cultures could have been caused by variations in the passaging and manipulation of the cultures, variations in the age of the animals used to harvest bone marrow, or variations in the GM-CSF used in the culture media. Variations in the passaging and manipulation of the cultures refer to individual differences in how each investigator handles the passaging and harvesting of the BMDC. While

every attempt was made to keep the passaging and harvesting of the BMDC the same for every experiment, different equipment and different time frames of passaging were occasionally used. Variations in the age of the animals used to set up the BMDC cultures refers mostly to the culture of TAP-/- BMDC as some of the TAP-/- mice used to harvest bone marrow were as much as 4 months older than the wild type mice. The concentration of GM-CSF used in the BMDC culture media could also have varied because the source of GM-CSF was supernatant from a GM-CSF-producing hybridoma. Once again, care was taken to use the GM-CSF from the same GM-CSF harvest, thereby keeping the concentration of GM-CSF constant throughout an experiment. Future experiments should eliminate some of these sources of variation by using age-matched animals under the age of 6 months for harvesting bone marrow and using known concentrations of GM-CSF.

6.2 Clinical Implications

The infectious diseases that currently pose the greatest threat to humans, AIDS, tuberculosis and malaria have all been resistant to traditional empirically derived vaccination strategies. AIDS, tuberculosis and malaria are likely to require a vaccination strategy designed to elicit T cell-mediated immunity. The design of vaccines to stimulate T cells requires a mechanistic understanding of how T cells are activated *in vivo*. Our findings suggest that rational vaccine design should take protein stability into account when targeting proteins to the direct or cross-priming pathways. Vaccines seeking to target the direct priming pathway should use newly synthesized, rapidly degraded proteins. The vector used to express the antigen(s) targeted to the direct priming pathway should ideally not inhibit protein synthesis as protein synthesis of cellular factors as well as neosynthesis of antigen were demonstrated to be required for direct presentation. Vaccines seeking to target the cross-priming pathway should utilize long-lived

stable protein. While cross-priming is a passive process, research from other groups has demonstrated that there is a threshold of antigen expression in order for cross-priming to occur (Hou et al., 1994; Kundig et al., 1996). Thus, vaccines targeting the cross-priming pathway also need to expresses sufficient antigen to permit cross-priming to occur. At first glimpse, this threshold of antigen expression for cross-priming to occur seems to explain our results in Chapter 4. Because we demonstrate that rVV expresses at least 1000-fold more OVA than rVSV, it may seem logical to conclude that rVV-expressed OVA can access the TAP-independent cross-priming pathway simply because rVV expresses sufficient OVA to overcome the threshold requirements of this pathway. However, in our reconstitution experiments, where cells were electroporated with OVA and then infected with parental (non-OVA expressing) strains of rVV or rVSV, the amount of OVA was constant yet rVV infection still permitted OVA access to the TAP-independent cross-priming pathway while rVSV and mock infection did not. Our findings from our experiments with rVV and rVSV also emphasize the importance of choosing the right vector for rational vaccine design. As a vector, rVV expression permitted the antigen to access an antigen processing pathway it normally is excluded. Thus, expression by rVV may enhance presentation of antigens. However, despite the fact that rVV seems like the better vector because it allows the antigen to access more antigen presentation pathways, rVSV-expressed OVA stimulated 5 times more CD8+ T cells than rVV-expressed OVA. Previous studies have demonstrated the importance of the number of T cells stimulated in the primary immune response on the memory T cell response (Albert et al., 1998b; den Haan and Bevan, 2002; den Haan et al., 2000; Mitchell et al., 1998; Pooley et al., 2001; Rock et al., 1990; Schulz and Reis e Sousa, 2002). Thus, rVSV might actually be the better vaccine vector. Another consideration to vaccine design brought up by our results is that the viral vector used to express the antigen also affects the antigen presenting cells needed to stimulate an antigen-specific T cells response. This consideration is more than just that of tissue tropism. While tissue tropism is already a

consideration, the requirement for specific populations of antigen presenting cells is relatively unknown and only just beginning to be probed. The ability to cross-prime antigens has already been demonstrated to vary between cell subsets and/or anatomical locations (Ritter et al., 2002; Zhu et al., 2007). Further investigation is imperative to determine how best to target vectors to specialized antigen presenting cells to induce an effective antigen-specific immune response.

The results from our studies also have clinical implications for the field of gene therapy. Gene therapy consists of techniques for correcting defective genes responsible for disease. To correct the defective gene, the therapeutic gene needs to be inserted into the genome and expressed as a functional protein. Because viruses have evolved to deliver their genetic material into cells, they are currently the preferred method for delivering therapeutic genes to their target cells. Gene therapy viral vectors are engineered to infect the target tissue and deliver the therapeutic gene without replicating. Despite the removal of viral genes that promote pathogenesis, gene therapy viral vectors still retain significant immunogenicity (Benigni et al., 2006; Zhu et al., 2007). Although many groups are experimenting with adding immunomodulatory proteins to gene therapy viral vectors (Albert et al., 1998b; Bosnjak et al., 2005), our findings that the donation of antigen to cross-priming is a passive process implies that even if direct priming of the transgene is prevented, cross-priming can still cause treated cells to be targeted for destruction. Furthermore, as gene therapy vectors generally deliver the therapeutic gene under the control of a strong promoter, such as the CMV promoter, it is likely that the protein expression of the therapeutic gene will be high enough to pass the threshold required for cross-priming to occur. Non-viral vector delivery systems for gene therapy consist of liposomes and DNA vectors targeted to specific cells by a bound protein receptor. DNA vectors and liposomes are immunogenic and activate the innate immune system; setting up inflammatory conditions that favor cross-priming rather than cross-tolerance. However, considering our finding that rVSV expressed transgenes activate transgene-specific T cells only

when the proper antigen presenting cell subsets are available, gene therapy may still be possible if the specific antigen presenting cell subsets are ablated prior to gene therapy treatment.

6.3 Future Directions

While our findings have shed some light on the requirements of the cross-priming pathway *in vivo*, several questions concerning this pathway must still be answered. We demonstrated that the form of antigen transferred in cross-priming is stable, long lived protein. Yet how the long-lived protein is transferred to the antigen presenting cell remains unknown. Our experiments do not address whether the antigen is transferred as part of an exosome or as part of a necrotic or apoptotic cell. As dendritic cells have been demonstrated to cross-present both apoptotic (Sauter et al., 2000) and necrotic cells (Neijssen et al., 2005), it is conceivable that a long-lived stable protein could be transferred to an antigen presenting cell via apoptotic or necrotic cells. Alternatively, the Neefjes group has demonstrated that polypeptides can be transferred to an antigen presenting cell via gap junctions (den Haan et al., 2000; Iyoda et al., 2002; Pooley et al., 2001). While this contradicts our results which demonstrate that cross-priming favors the transfer of long-lived stable protein, the Neefjes group demonstrates that there is more than one pathway for transfer of antigen. Additionally, more research is needed into how and when antigen presenting cells use the cross-priming pathway. While CD8+ dendritic cells are implicated as being more efficient at cross-priming T cells than other antigen presenting cell subsets (Kurts et al., 1998; Kurts et al., 1999), it is conceivable that different antigen presenting cell subsets are used under different conditions or anatomical locations. It is also conceivable that different subsets of antigen presenting cells could cross-prime different types of antigen. Another question remaining to be answered about the cross-priming pathway is what factors

determine whether you presentation of transferred antigen results in cross-priming (T cell activation) or cross-tolerance (T cell tolerance). There is evidence that high concentrations of antigen result in cross-priming while low concentrations of antigen result in cross-tolerance (Kurts et al., 1999). However, introduction of inflammatory conditions abrogated the need for high concentrations of antigen (Hou et al., 1994; Kundig et al., 1996). A better understanding of conditions that favor cross- tolerance would have implications for cancer therapy, gene therapy, graft tolerance and autoimmune disease.

Our findings that the vector used to express the antigen can affect the antigen processing and presentation pathways available to the antigen leads to several directions for future vaccine vector research. First, can rVV expression lead to TAP-independent cross-priming for other TAP-dependent antigens. While we have demonstrated that some intrinsic factor of rVV infection permits OVA to access the TAP-independent pathway, it remains to be determined if rVV infection will alter the antigen processing pathways available to other antigens. In addition, it remains to be determined what, if any, effect this enhanced access to antigen processing and presentation pathways plays on the development of T cell memory. Figure 4.2 demonstrates that rVSV-expressed OVA produces 5-fold more IFN-γ producing T cells than rVV-expressed OVA. Previous studies have demonstrated the importance of the number of T cells stimulated in the primary immune response on the memory T cell response (Zaks et al., 2006), it would be interesting to examine which of the vectors activated the best OVA-specific T memory response. Additionally, as mentioned in the Chapter 5 discussion, determining whether ablation of splenic red pulp macrophages and/or B cells ablates not only VSV-specific T cells activation but OVA-specific T cells activation. If ablation of antigen presenting cell subsets ablates presentation of the transgene, it will add another layer of complexity to the design of vaccine vectors.

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