MACROPHAGES DIRECTLY PRIME NAÏVE $CD8^+$ T CELLS

A Dissertation Presented

By

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ABSTRACT

Professional antigen presenting cells (APCs) represent an important link between the innate and adaptive immune system. Macrophages (MΦs) and dendritic cells (DCs) serve as sentinels in the periphery collecting samples from their environment and processing this information. These cells then present antigenic fragments to T cells in the context of self-MHC molecules. Although a clear role for both of these APCs in the stimulation of already activated or memory T cells has been established, the ability of MΦs to activate naive T cells is still unknown. In this thesis the ability of bone marrow-derived MΦs and DCs to prime naive $CD8^+$ and $CD4^+$ T cells was investigated. Using adoptively transferred transgenic CFSE-labeled P-14 T cells, specific for gp33 from lymphocytic choriomeningitis virus in the context of D^b, we were able to demonstrate the ability of both MΦs and DCs to induce naive $CD8^+$ T cells proliferation. Once primed by MΦs these T cells gained effector function as shown by interferon- γ (IFN-γ) production and in vivo cytolysis. In addition, immunization of wild type animals with gp33-pulsed MΦs, as well as DCs, led to greater than a 95% reduction in lymphocytic choriomeningitis virus titers. To rule out the role of cross-presentation in the observed priming, two models were used. In the first model, lethally irradiated F1 bxs chimeras reconstituted with either $H\text{-}2^s$ or $H\text{-}2^b$ bone marrow were used as host for the adoptive transfer experiments. Since the gp33 peptide binds to D^b, the $H\text{-}2^s$ reconstituted animals should be unable to cross-present the peptide to the P-14 T cells. Using this model, we were able to clearly demonstrate the ability of MΦs to activate naive P-14 T cells to undergo division. Additional experiments, demonstrated that these MΦ primed T cells

went on to develop into effector cells. Finally, the ability of the MΦ primed T cells to develop into functional memory cells was demonstrated. To confirm the chimera results, these experiments were repeated using β2 microglobulin deficient animals (whose cells don't express MHC I) as host in adoptive experiments. MΦs were able to stimulate the naive P-14 T cells to divide and gain effector function as demonstrated by the ability to produce IFN-γ. In contrast to the CD8 system, MΦ were poor stimulators of DO11.10 $CD4^+$ T cell proliferation. Additionally, DO11.10 T cells stimulated by DCs were able to produce interleukin-2 (IL-2), IL-4, tumor necrosis factor and granulocyte-macrophage colony stimulating factor where as MΦ stimulated DO11.10 T cells were only able to produce IL-2. In conclusion this body of work clearly demonstrates the in vivo ability of MΦ to stimulate $CD8^+$ T cell proliferation, effector function, as well as the formation of functional $CD8^+$ T cell memory. Whether or not the nature of the memory pools stimulated by the two APCs is exactly the same is still unknown and needs further investigation. The ability of APCs other than DCs to stimulate functional protective memory needs to be considered in the quest to design vaccines that offer broad-spectrum protection.

TABLE OF CONTENTS:

TABLE OF FIGURES:

TABLE OF TABLES:

Abbreviations Used In This Thesis:

Ag	antigen
APC	antigen presenting cell
BM	bone marrow
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DC	dendritic cell
FCS	fetal calf serum
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN-γ	interferon-gamma
IL	interleukin
IV	intravenous
gp33	KAVYNFATC
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
LN	lymph node
M-CSF	macrophage colony stimulating factor
MHC I	major histocompatibility complex I
MHCII	major histocompatibility complex II
MLR	mixed leukocyte reactions
MΦ	macrophage
Nag	no antigen
OVA	ovalbumin
OVA-p	SIINFEKL
SC	subcutaneously
TAP	transporter-associated with antigen presentation

Chapter I: Introduction

The ability of an organism to fight off infection by other organisms is dependent on its immune system. The immune system consists of two parts, innate immunity and adaptive immunity. Innate immunity evolved first, appearing in invertebrates such as *Drosophila* and *C. elegans*. In the vertebrates, innate immunity acts as the first line of defense against infection. Some components of the innate immune system are complement, interferons α/β, natural killer cells (NK) and the mononuclear phagocyte system including dendritic cells, macrophages and granulocytes. The phagocytes of innate immunity can be activated by the binding of the pathogens to the pattern recognition molecules, Toll-like receptors, on their surface. These cells are also activated by the inflammatory cytokines. Activated phagocytes then engulf the invading organism and destroy it using microbicidal molecules including reactive oxygen intermediates. Since mammalian cells generally do not express the molecular patterns that the Toll-like receptors recognize, they signal the immune system that the host is being invaded. The innate immune system recognizes unprocessed antigens such as carbohydrates (Poltorak et al., 1998), double stranded RNA (Alexopoulou et al., 2001) and DNA (Hemmi et al., 2000). In this manner innate immunity is signaled to the presence of a foreign invader by receptors with limited numbers of specificities (Akira, 2003; Takeda et al., 2003).

Adaptive immunity first appeared in the vertebrates and consists of the lymphocytes, B and T, which respond in an antigen specific manner. Both B and T cells possess surface antigen receptors that undergo gene rearrangements and selection during development (LeFranc et al., 1986; Reth and Alt, 1984; Seide and Kehoe, 1983;

Waldmann et al., 1985). The various stages of gene rearrangements and the process that allows for the gene breaks to be repaired generate the tremendous variability in both the T cell and B cell repertoire. This large variability in the repertoire results in an organism's ability to recognize any pathogen and ward off infection. Although B cells can recognize and bind whole antigens, T cells respond only to antigenic fragments displayed in the correct context. The cells responsible for the initial processing and correct presentation of antigen to the adaptive immune system, thereby alerting it as to whether or not an immune response is needed are of bone marrow origin (Beutler, 2004). While it has been thought that dendritic cells are the key cells that carry out this surveillance function, this thesis demonstrates that macrophages also carry out this important function. In this process macrophages and dendritic cells act as sentinels in the periphery collecting samples from their environment and processing this information. These phagocytes then display antigenic fragments on their cell surface in the context of the correct major histocompatibility complex. These cells also express surface molecules such as CD80 and CD86 that aide in their ability to activate T cells. Both macrophages and dendritic cells secrete various cytokines and chemokines to further attract and activate T cells. By collecting, processing and presenting antigens, macrophages and dendritic cells allow for the transfer of antigenic information from the innate immune system to the adaptive immune system. Antigen presenting cells (APC) are the cells that serve as the link between the two components of the immune system and are the subjects of this thesis.

Antigen Processing and Presentation

Endogenously synthesized antigen and MHC Class I

Class I molecules are found on all nucleated cells in the body. Classical Class I molecules consist of heavy chains and β2 microglobulin (β2m). The β2m molecule aids in the folding and the stabilization of the class I molecule. The class I pathway samples cellular proteins that are targeted to the proteosome for degradation (Gaczynska et al., 1993; Goldberg and Rock, 1992). The proteosome generates peptides with the correct C terminus, but various extensions on the N-terminus (Mo et al., 1999). These peptides then bind to the transporter associated with antigen processing (TAP) protein and are transported into the endoplasmic reticulum (Monaco, 1992) where N-extended precursors can be trimmed by amino peptidases (Mo et al., 1999). The mature epitopes bind to the newly formed class I, β2-microglobulin complex, and then these new class I molecules follow the default exocytosis pathway to be displayed on the cell surface (Rock et al., 2004; York et al., 1999; York and Rock, 1996).

Exogenous Antigen And MHC Class I

Another pathway for the generation of peptides for class I exists exclusively in professional APCs (Huang et al., 1994a; Huang et al., 1994b; Sigal et al., 1999; Sigal and Rock, 2000). This pathway is called the exogenous class I pathway or cross-presentation pathway (Bevan, 1976a; Bevan, 1976b). Antigens destined for this pathway enter the APCs through endocytic mechanisms e.g. phagocytosis of debris or apoptotic blebs from infected cells. Once in the phagolysosomes, there are two possible fates for these antigens: a TAP- dependent (Huang et al., 1996; Song and Harding, 1996) and TAP-

independent pathway (Bachmann et al., 1995; Sigal and Rock, 2000). In the TAP-dependent pathway, the mechanism by which the peptides exit the phagosome and enter the cytoplasm is largely unclear, but might involve Sec 61(Tsai et al., 2002). Once in the cytoplasm these antigens are broken down by the proteosome into peptide fragments. These peptides are then transported into the ER via the TAP transporter and loaded onto newly synthesized class I molecules. In contrast, the TAP-independent presented peptides are generated in the endocytic compartment, and this process has recently been shown to be dependent on Cathespsin S (Rock, 2003; Shen and Rock, 2004). These cross-presentation pathways have been shown to play a very important role *in vivo* in the immunity to viruses and pathogens that do not infect professional antigen presenting cells and possibly in all infections (Belz et al., 2004; den Haan and Bevan, 2001; Lenz et al., 2000; Rock, 2003)

Loading Of Class II MHC Molecules

In contrast to class I molecules, class II molecules are only found on professional APCs. Class II molecules are synthesized as a heterodimer consisting of α and β chains (Neefjes and Ploegh, 1992). Invariant chain associates with the $\alpha\beta$ heterodimer and stabilizing its structure, blocking its peptide binding groove and directing its transport. In the endocytic compartment cysteine and aspartic proteases digest the invariant chain leaving a peptide fragment in the peptide-binding groove termed CLIP (Class-II-associated invariant chain peptide). Antigens destined to be presented on class II molecules enter the APC through endocytosis, pinocytosis or phagocytosis (Harding et al., 1991; Harding and Song, 1994; Neefjes et al., 1990). After internalization, the

antigen is broken down by the proteases present in the endocytic compartments such as the cathepsins (Driessen et al., 1999; Pluger et al., 2002). In antigen presenting cells there is a specialized endosomal/lysosomal compartment termed MHC class II compartment (MIIC), where the CLIP peptide is exchanged for an antigenic peptide (Peters et al., 1991) . The CLIP peptide can also be removed in a compartment called CIIV, which is a multivesicular compartment that more closely resembles endosomes (Amigorena et al., 1994; Amigorena et al., 1995). The accessory molecule H-2DM catalyzes this peptide exchange. These molecules then travel to the surface via the default exocytosis pathway. The peptides that bind to class II are generally longer than those that bind class I, about 12 amino acids long (Nelson et al., 1993; Nelson et al., 1992; Rudensky et al., 1991). Class II molecules can also be loaded after being recycled from the cell surface (Sinnathamby and Eisenlohr, 2003). Class II antigen processing and presentation is reviewed in Bryant *et al.* (Bryant and Ploegh, 2004).

Professional Antigen Presenting Cells

B cells, dendritic cells (DCs) and macrophages (MΦ) are all professional antigen presenting cells (APCs). Although all nucleated cells express class I and can present peptide on MHC class I molecules to $CD8^+$ T cells, only professional APCs can provide the T cells with the necessary costimulatory signals leading to T cell stimulation. Professional APCs (specifically MΦ and DCs) are the only cells that have the ability to present exogenously acquired antigens on class I molecules (Harding and Song, 1994; Rock et al., 1993). This specialized function is termed cross-presentation and is

important in immunity to viruses that don't infect APCs and in tumor surveillance (see above) (Belz et al., 2004; den Haan and Bevan, 2001; Lenz et al., 2000; Rock, 2003). In addition, a professional APC has all the necessary molecules for the efficient antigen presentation to T cells including MHC class I, MHC class II, costimulatory molecules B71/B72 and CD40 (Banchereau et al., 1995). Professional APCs also express adhesion molecules and chemokine receptors, which allow them to home to the secondary lymphoid tissue (Banchereau and Steinman, 1998). DCs and MΦs resident in the tissues or circulating in the blood gather antigenic information from the local environment and then migrate to the secondary lymphoid organs (Matsuno et al., 1996). APCs that reside in the lymph nodes and spleen may also acquire antigen from lymph fluid or blood, respectively. These cells then generate peptides from the antigens they have acquired and display them on their MHC class I and class II molecules. Once in the lymphoid tissue, the APCs will present these peptide-MHC class I and class II complexes and costimulatory molecules to naive T cells and initiate an immune response. The main job of these cells is to act as sentinels in the periphery (Austyn, 1996; Banchereau and Steinman, 1998)

B cells as APCs

B cells have the ability to be both APCs and antibody-secreting cells. Although B cells typically acquire and present soluble antigens, they can in some circumstances also present particulate antigen on Class II. Using T-T hybridomas as a read out, Vidard *et al.* demonstrated that B cells can present particulate antigens on MHC II very effectively when activated by LPS and that presentation is enhanced if the B cells immunoglobulin

receptor can bind the antigen. These authors went on to show that the epitopes presented by the B cells differed from the epitopes presented by the MΦs (Vidard et al., 1996). Another study suggests that entry of antigen into the B cell after binding to the immunoglobulin receptor may lead to an accelerated processing pathway for Class II containing novel compartments (Cheng et al., 1999).

B Cells Can Present Antigen To Naive T Cells In Vitro

There is abundant literature on the ability of B cells to present antigen and stimulate previously activated $CD4^+$ T cells *in vitro* (Ashwell et al., 1985; Bottomly and Janeway, 1989; Duncan and Swain, 1994; Kurt-Jones et al., 1988; Liano and Abbas, 1987; Lichtman et al., 1987; Linton et al., 2003; Malynn et al., 1985; Morokata et al., 1995; Webb et al., 1985; Webb et al., 1989; Zimecki et al., 1988). It has also previously been shown that small B cells can stimulate mixed lymphocyte reactions as well as Mlsa –reactive T cells (Webb et al., 1985; Webb et al., 1989). The ability of resting B cells to present antigen to $CD4^+$T cells *in vitro* has also been previously demonstrated (Liano and Abbas, 1987; Lichtman et al., 1987). In addition, activated (e.g. anti-Ig and IFN-γ) stimulated B cells express B7.2, HSA and ICAM-1 (Morokata et al., 1995). Therefore activated B cells express many of the molecules needed to activate naive T cells, making them potent stimulators of T cell responses. These anti-μ and IFN-γ stimulated B cells were able to stimulate naive $CD4^+$ T cells to proliferate (Morokata et al., 1995). Blocking experiments demonstrated that the expression of B7.2 was critical for the B cells to function as APCs. Although the naive cells responded to this stimulation,

$CD45RB_{low}$ memory cells proliferated minimally, suggesting that B cells may be more potent stimulators of naive T cells than of memory T cells (Morokata et al., 1995).

Using transgenic mice specific for a peptide derived from pigeon cytochrome C, Duncan *et al.* showed that both isolated activated B cells and bone marrow-derived MΦs had the ability to stimulate naive $CD4^+$ T cells to become Th effector cells *in vitro*. The ability to skew the effector population with exogenous IFN-γ or IL-4 was irrespective of the stimulating APC. MΦs and B cells were equally capable of restimulating Th1 or Th2 effector cells. These authors went on to a note a difference of the 2 types of APC in their ability to skew effector Th0 development. Whereas stimulation of T cells with B cells led to secretion of more IFN-γ, stimulation of effector Th0 cells with MΦs led to secretion of more IL-4 (Duncan and Swain, 1994).

B Cells Can Present Antigen To Naive T Cells In Vivo

Using mice lacking B cells, Constant *et al.* demonstrated a role for B cells in the presentation of protein antigens. These mice were still able to mount responses to peptide antigens. This group went on to further demonstrate that B cells pulsed with protein antigen *in vivo* up-regulated expression of B7.2 (Constant et al., 1995b). Yet another study performed by Ron *et al.* also demonstrated a role for B cells in T cell priming. Using mice that lack Ig^+ B cells and are unable to mount T cell proliferative responses; these authors showed that this defect could be rescued by the injection of purified B cells one day before administration of peptide and adjuvant (Ron and Sprent, 1987). These data suggest a clear role for B cells in T cell priming, but a conflicting report by Fuchs *et al.* demonstrated that although B cells can present antigen to memory

T cells, they are unable to prime naive T cells (Fuchs and Matzinger, 1992). A more recent study demonstrated that B cells could present antigen to $CD4^+$ T cells *in vivo*. These authors also showed that costimulation via OX40L leads to the development of Th2 cells. These $CD4^+$ T cells are able to expand and secrete cytokines (Linton et al., 2003). Taken together these studies demonstrate that under the right conditions with proper activation B cells can present antigen efficiently to naive T cells. In addition the stimulation of $CD4^+$ T cells by different types of APCs may determine the effector cytokines made by those $CD4^+$ T cells.

Dependence on CD40-CD40L

The ability of B cells to stimulate naïve $CD4^+$ T cells is dependent on B cell activation. Ligation of CD40 on B cells appears to be important for activating B cells whereas priming of T cells by naïve B cells frequently leads to the induction of tolerance. CD40 is a member of the tumor necrosis factor (TNF) superfamily of receptors and is expressed on professional APCs, epithelial cells, fibroblast and mast cells among others. The ligand for CD40, CD40L also known as CD154 is expressed on T cells, NK cells, mast cells and other cell types. The ligation of CD40 by CD40L on Th2 cells is critical in the activation of B cells and antibody responses. The engagement of CD40 by its ligand leads to the up regulation of CD80, CD86, and CD54 (LFA-1) as well the secretion of various cytokines and chemokines by the B cells. B cells activated by CD40-CD40L interactions secrete IL-1, IL-6 TNF-α, IL-10 and IL-12, which are important mediators of immune responses (Van Gool et al., 1996; Van Kooten and Banchereau, 1996; van Kooten and Banchereau, 1997a; van Kooten and Banchereau, 1997b; van

Kooten and Banchereau, 1997c; van Kooten and Banchereau, 2000). Mice deficient in CD154 have reduced proliferative responses to protein antigens, are unable to mount CTL and do not develop autoimmune diseases in some model systems. Inhibition of CD40-CD154 interactions, either by blockade or genetic knockout, blocks humeral responses to T dependent antigens (responses dependent on Th2 help), graft versus host disease and rejection of tissue allografts (Boussiotis et al., 1996; Boussiotis et al., 1994). Some of these observed defects may be due to the absence of CD40 on other APCs such as DCs and MΦs.

Using splenic APCs from CD40-deficient and wild type mice to prime $CD4^+$ T cells expressing the transgenic TCR for ovalbumin (DO11.10 cells) *in vitro*, Ozaki *et al.*, showed that $CD40^{-/-}$ splenocytes were able to induce good proliferation of the $CD4^+$ T cells to ovalbumin, concluding that in the presence of high peptide concentration the ability of splenic APCs to stimulate T cell responses is independent of CD40-CD154 interactions. These authors went on to show that, when resting B cells were isolated from the CD40-deficient mice, the B cells were unable to stimulate naïve T cells. Using super antigens to prime naive T cells, the authors confirmed the ability of the B cells from knockout animals to present strong antigenic stimuli to the T cells (Ozaki et al., 1999). In conclusion, although naive B cells can present high dose antigens, only B cells activated through the B cell receptor and CD40 ligation are able to stimulate naive T cells when antigen is limiting.

The Role Of B Cells In Cross-presentation

The ability of B cells to cross-present exogenous antigen on class I molecules is limited. In studies isolating MΦs and B cells from murine spleens and testing their ability to present peptides from ovalbumin to CTL clones, Rock *et al.* demonstrated that B cells were unable to present antigens via this pathway (Rock et al., 1993). On the other hand, a recent study has shown that B cells are able to cross-present bead bound ova to $CD8^{+}$T cells if the bead was also conjugated to CpG-DNA (Heit et al., 2004). The ability of B cells to cross-present antigen when activated via the toll receptor pathways highlights the differences in the ability of resting and activated B cells to efficiently cross-present antigen.

Dendritic Cells

DCs are professional APCs that reside in most tissues in the body and act as sentinels in the immune system. In the peripheral tissues, DCs are constantly "sampling" the environment and internalizing antigens. DCs then process and present these antigens to T cells. The ability to process and present both self and nonself antigens allows DCs to play a role in both the initiation of T cell activation and in T cell tolerance and anergy.

In the tissues DCs exist in an immature state in which they are highly phagocytic and express only low levels of MHC class II and the costimulatory ligands CD80 and CD86. Once DCs acquire antigen and receive activation signals, e.g. from CD40-CD40L interactions, Toll receptor signals and/or exposure to inflammatory cytokines, they begin a maturation process and migrate to the lymph nodes (Denzin et al., 1996; Reis e Sousa, 2004a; Reis e Sousa, 2004b). After the DCs mature, they are no longer

phagocytic, but express high levels of MHC Class II molecules (Pierre et al., 1997) (Cella et al., 1997a), CD80, CD86 (Caux et al., 1994; Inaba et al., 1995; Inaba et al., 1994) and secrete various cytokines such as IL-12 (Cella et al., 1996). In this mature state DCs are found in the T cell zones of the secondary lymphoid organs where they are able to interact with and activate naïve T cells.

All DCs arise from bone marrow precursor cells. Possibly due to the importance of their function, DCs are a very heterogeneous population of APCs. Since a marker specific to the dendritic cell lineage has yet to be identified, DCs are currently defined by varying criteria including lineage, surface markers, maturation, anatomical location and ability to present antigen to naive T cells. How all these subpopulations relate to one another is still not entirely clear. Mouse spleens contains 3 major subsets of DCs distinguished by the expression of various cell markers $CD8^+$ DEC 205^+, $CD4^+$ and $CD4^-$ $CD8^-$. Whereas mouse lymph nodes contain 6 known subsets: $CD8^+$ $CD205^{high}$ $CD11b^+$ (Langerhan cells); $CD8^+$ $CD205^{high}$ $CD11b^-$ (lymphoid DCs); $CD4^+$ $CD11b^+$; $CD4^-$ $CD8^-$ $CD205^+$ $CD11b^+$; $CD4^-$ $CD8^-$ $CD205^-$ $CD11b^+$ and $CD45RA^+$ ($B220^+$) (plasmacytoid DCs) (Belz et al., 2002c; Henri et al., 2001; Kamath et al., 2000; Vremec and Shortman, 1997).

Generally, DCs can be grouped into 2 major subsets based on their phenotype: lymphoid DCs ($CD8^+$) and myeloid DCs ($CD8^-$ $CD4^+$). The lymphoid DCs are $CD8\alpha$ homodimer positive, $CD11c^+$, $DEC205^+$ and $CD11b^+$. Lymphoid DCs are thought to be less phagocytic than myeloid DCs and produce higher levels of Interleukin-12 (IL-12), upon stimulation. In response to IL-12 lymphoid DCs can produce IFN-γ (Banchereau

and Steinman, 1998). Myeloid DCs arise from a common myeloid precursor in the presence of Granulocyte/macrophage colony-stimulating factor and are characterized by the expression of CD11b and CD11c but lack CD8α and DEC205. Bone marrow-derived, $CD11b^+$ $CD11c^+$ myeloid DCs are the subject of this thesis. Which of the above-mentioned *in vivo* populations this *in vitro* raised population of DCs represents is still unknown and is currently under investigation.

In Vitro Evidence For The Ability Of DC To Present Antigen To $CD4^+$ T Cells

There is abundant literature supporting a role for DCs in the *in vitro* stimulation of $CD4^+$ T cells. One of the first observations made about the immunostimulatory role of DCs was their ability to induce primary mixed leukocyte reactions (MLR) in mice. Using splenic DCs, Steinman *et al.* showed that as few as 300-1000 DCs could stimulate very potent proliferative activity of responding splenic T cells and that 100,000 DCs was able to induce maximum T cell proliferation of 30-80 fold. DCs were also shown to have 100-300 times more MLR-stimulating ability than unfractionated spleen cells. In addition, depletion of splenocytes of T or B cells had no affect on the ability of these cells to stimulate MLR. This group further demonstrated that DC-depleted cell populations that were enriched for mononuclear phagocytes were able to stimulate MLRs very weakly (Steinman et al., 1983; Steinman and Witmer, 1978). Another early paper showed that DCs were also able to stimulate unprimed syngeneic T cells to proliferate in the absence of antigen. This activity is termed a syngeneic mixed leukocyte reactions. It was further demonstrated that the ability of DCs to stimulate alloreactive T cells was 10-fold greater than their ability to stimulate syngeneic T cells (Nussenzweig and Steinman,

1980). In addition, Inaba *et al.* demonstrated that Bacillus Calmette-Guerin (BCG) - pulsed DCs could induce proliferation *in vitro* of BCG-specific T cells isolated from animals immunized with BCG and an adjuvant (Inaba et al., 1993).

In Vivo Evidence For The Ability Of DC To Present Antigen To $CD4^+$T Cells

Using dye labeled DO11.10 $CD4^+$ transgenic T cells (specific for the OVA peptide 323-339) and an adoptive transfer system, Ingulli *et al* demonstrated clustering of transgenic T cells around dye labeled peptide-pulsed APC. Additionally, this group demonstrated the ability of the transgenic T cells to expand and secrete IL-2 in the mice that received the adoptively transferred peptide-pulsed DCs. In further experiments, the ability of the primed $CD4^+$ T cells to initiate a delayed type hypersensitivity response was demonstrated (Ingulli et al., 1997). In another set of experiments by this same group, a role for $CD11b^+$ DC in the presentation of soluble OVA to $CD4^+$ T cells was demonstrated (Ingulli et al., 2002). In these experiments, DO11.10 T cells were adoptively transferred into BALB/c hosts. Twenty-four hours later, the mice were inoculated subcutaneously with fluorochrome labeled OVA. When the draining lymph nodes were removed eighteen hours later and analyzed by FACS, $CD8^+$ DCs, a sub population of B cells and MΦ contained low amounts of OVA, whereas a subpopulation of $CD11b^+$ DCs contained large amounts of OVA. This group went on to show that only $CD11b^+$ DCs sorted from soluble OVA injected mice could stimulate DO11.10 T cells to proliferate *in vitro.* The inability of the $CD8^+$ DC to present OVA *in vivo* is interesting, since when given soluble OVA *in vitro* the $CD8^+$ DC were able to generate the correct peptide-MHC complexes and present antigen to T cells. Since most of the $CD11b^+$ DCs

are in the paracortical region near the B cell region and the $CD8^+$ DCs were equally distributed throughout the paracortex, the authors suggested that one possible explanation for this discrepancy might be that subcutaneously delivered soluble antigen is sequestered in certain compartments (Ingulli et al., 2002). This observation suggests that the type of antigen as well as the route of entry into the body may determine the most efficient APC for antigen presentation to T cells.

Studies involving immunity to intracellular parasites also demonstrate a role for DCs in the presentation of antigen to $CD4^+$ T cells. Flohe *et al.* demonstrated that immunization with Langerhan and DCs pulsed with *Leishmania major in vitro* could protect BALB/c mice from a lethal challenge whereas; immunization with *L. major* pulsed MΦs did not offer protection (Flohe et al., 1998; Moll and Flohe, 1997). Since immunity to *L. major* had previously been shown to depend on the activation of $CD4^+$ T cells, activation of these cells was inferred from the observed protection (Erb et al., 1996). A role for antigen pulsed DCs in immunity to mycobacteria has also been demonstrated. Inaba *et al.* showed that BCG-pulsed BM-derived DCs could induce immunity to BCG in naive mice. By antibody depletion and blocking studies, this group further demonstrated that the proliferating cells isolated from these mice were $CD4^+$ T cells (Inaba et al., 1993).

In Vitro Evidence For The Ability Of DC To Present Antigen To $CD8^+$ T Cells

Although proliferation of T cells in a MLR is thought to be primarily proliferation of $CD4^+$ T Cells, $CD8^+$ T cells are also induced to proliferate in this system. Steinman *et*

al. also showed that during a MLR, DCs were also able to induce alloreactive cytotoxic T cells (CTL), clearly demonstrating a role for DCs in the *in vitro* priming of $CD8^+$ CTL (Steinman et al., 1983).

Using DCs exposed to exogenous antigen from cytomegalovirus (CMV), two groups have demonstrated that DCs can process and present viral epitopes to human $CD8^+$ T cells, leading to expansion of CMV-specific T cells. Once expanded, the CTLs were able to lyse peptide-pulsed targets *in vitro* (Kleihauer et al., 2001; Peggs et al., 2002). Transfusion of the T cells activated and expanded by peptide-pulsed DCs into transplant patients, in which CMV has reactivated, leads to a reduction in viral burden (Einsele et al., 2002). Others have shown that human DC's pulsed with known CTL epitopes can present antigen to both naive and memory CTL (Wong et al., 1998).

In Vivo Evidence For Ability Of DC To Present Antigen To $CD8^+$T Cells

Although it is presently thought that DCs are the only cells that can stimulate naive T cells *in vivo* (Banchereau and Steinman, 1998), experimental evidence for this concept is largely lacking (see below). There is considerable evidence that DCs can acquire and present antigen *in vivo*. By transferring *in vitro* OVA-loaded β2m-deficient splenocytes into wild type animals, den Hann *et al.* demonstrated that $CD8^+$ DCs could cross-present OVA and prime $CD8^+$ CTL (den Haan et al., 2000). Similarly, Pooley *et al.*, using intravenously administered soluble OVA, demonstrated a role for $CD8^+$ DCs in the presentation of soluble OVA to $CD8^+$ T cells. However, in the same study, a role for $CD8^-$ DCs in the presentation of OVA to $CD4^+$ T cells was clearly established (Pooley et

al., 2001). Many other experiments have shown that in immunized animals, DCs that are presenting the injected antigen can be recovered.

There is also considerable data showing that injection of antigen-bearing DCs into mice can prime T cell responses. Many studies have tried to use DCs loaded with antigen *ex vivo* to initiate $CD8^+$ T cell responses *in vivo* against tumor antigens (Celluzzi et al., 1996; Gabrilovich et al., 1996; Paglia et al., 1996; Tjandrawan et al., 1998; van Broekhoven et al., 2004). One such study performed by Paglia *et al.*, used BM-derived DCs loaded *in vitro* with soluble Beta-galactosidase (β-gal) as a model. This group demonstrated that when transferred into BALB/c mice intraperitoneally, these antigen-loaded DCs were able to induce CTLs. In addition, antigen-loaded DCs could be used to immunize mice against β-gal. Once immunized, 60 % of these mice were protected from tumor challenge with β-gal expressing tumors. If the immunized mice were boosted with soluble β-gal 5 days after priming, they were completely protected from tumor challenge (Paglia et al., 1996). In another model system Porgador *et al.* found similar results. These authors found that a single immunization of mice with OVA peptide-pulsed DCs was sufficient to elicit a protective immune response against a challenge with an OVA expressing tumor (Porgador et al., 1996). In another study, these same authors demonstrated the priming of naive CTL in mice immunized with OVA peptide pulsed DCs (Porgador and Gilboa, 1995). A study by Gabrilovich *et al.* took these observations one-step further. These authors demonstrated a role for peptide-pulsed dendritic cell immunization in the rejection of established tumors in tumor bearing mice. These authors found that although a single immunization with mutant p53-pulsed DCs had no

effect on tumor size, repeated immunizations led to arrested tumor growth and prolonged survival. Additionally, these authors showed a switch in the Th1/Th2 profiles of the treated mice. Tumor bearing mice showed a predominately Th2 profile in cytokine secretion, whereas mice treated with peptide-pulsed DCs displayed a more Th1-type profile of cytokine secretion. Another interesting observation made by these authors is that only DCs made from the bone marrow of tumor bearing mice and not splenic DCs had an effect on tumor progression (Gabrilovich et al., 1996). Taken together, these studies show that immunization with peptide-pulsed DCs can lead to potent anti-tumor responses. In addition, they suggest a role for antigen source and APC type in the initiation of T cell responses.

A role for peptide-pulsed DCs in the induction of viral immunity has also been demonstrated. Studies using hepatitis B surface antigen particle-pulsed DCs transferred into syngeneic hosts demonstrated a role for DCs in the priming of CTL against hepatitis B proteins (Bohm et al., 1995). The authors also demonstrated that although CTL generation was suppressed in carrageenan-treated mice, which eliminates both MΦs and DCs, CTL activity was not suppressed in mice treated with dichloromethylene-diphosphonate liposomes, which would only eliminate MΦs (Bohm et al., 1995). It was further demonstrated that CTL responses could be restored by the injection of peptide-pulsed MΦs in carrageenan-treated animals. Ludewig *et al.* used DCs pulsed with the immunodominant epitope from LCMV's glycoprotein *in vitro* or DCs that constitutively express the same epitope to immunize mice (Ludewig et al., 1998). These authors demonstrated that mice immunized intravenously with either group of DCs were

completely protected from a lethal LCMV challenge. In addition, splenocytes from these mice displayed direct *ex vivo* cytotoxicity against peptide-pulsed targets by day four. The immunized mice were protected from day two till day 60 from infections in which homing of T cells into peripheral organs is critical for viral clearance. These data suggest that immunization by peptide-pulsed targets offers long lasting viral immunity (Ludewig et al., 1998). Although these studies all suggest that injected DCs efficiently prime strong $CD8^+$ T cell responses that offer protection from viral challenge, a role for host APC in the observed priming still cannot be excluded.

In Vivo Evidence For The Exclusive Ability Of DCs To Present Antigen To $CD8^+$T Cells

One recent study took advantage of transgenic animals that express the receptor for diphtheria toxin under the control of the CD11c promoter. As previously mentioned, many populations of DCs express the CD11c molecule, whereas MΦs and B cells do not. One caveat worth mentioning is that activated T cells also express CD11c. In these mice the injection of diphtheria toxin eliminates DCs from the animal. Using this approach, Jung *et al.* demonstrated that injection of diphtheria toxin blocked the generation of CTLs to some antigens, e.g. *Listeria monocytogenes* and *Plasmodium yoelii* (Jung et al., 2002). Another caveat in this study was the possibility that the ability of MΦs to stimulate T cells was impaired by the toxin, e.g. when MΦs ingested the toxin during phagocytosis of dying DCs. These data are among the strongest to show that DCs can play an essential role in stimulating T cell responses at least for certain antigens. However, whether this

is true for other antigens is unknown; *in vivo* some antigens may be preferentially presented on DCs while others are also presented on MΦs. It is also interesting to note that the two pathogens chosen by the authors rely heavily on cross-presentation for protective immunity (Lenz et al., 2000; Shen et al., 1998).

In a set of experiments designed to visualize the APCs that interacted with naive T cells *in situ*, Norbury *et al.*, infected mice with a vaccinia virus expressing EGFP and could detect infected (EGFP-expressing) DCs and MΦs *in situ*. When they transferred CFSE-dye-labeled virus specific T cells, they observed clustering of these $CD8^+$ T cells around EGFP-positive (infected) APC. Although MΦs made up > 60% of infected cells, clustering was observed only around the infected DCs. This observation was interpreted to show that only DCs were stimulating the T cells however T cell stimulation was being inferred, and what was actually measured, T cell-APC clustering, is influenced by strength of adhesion, chemokines and potentially other factors. In fact, DCs can cause T cells to cluster, even in the absence of specific antigen (Inaba and Steinman, 1986). This analysis would fail to detect single T cells that were stimulated and /or ones that detached from an APC. Moreover, a substantial component of the T cell response to vaccinia virus occurs through cross-priming (Norbury et al., 2001; Sigal et al., 1999) and the APCs involved in this process would not express EGFP from the vaccinia recombinants. These data clearly demonstrate that DCs can and do present antigens to naive T cells, but a role for MΦs in antigen presentation still cannot be definitively ruled out.

Changes in MHC Class I Processing and Presentation in Mature DCs

Very little is known about the changes in MHC Class I upon dendritic cell maturation. Taking advantage of the ability of the phagocytosis of bacteria by DCs to induce both functional and phenotypic maturation, Rescigno *et al.* demonstrated that MHC class II molecules were up regulated with faster kinetics (within 1 hour) than class I molecules. Using 14 day-old bone marrow-derived DCs from mice, Rescigno *et al.* have shown both an increase in Class I synthesis as well as a 3-fold increase (from 3 to 9 hours) in stability of newly synthesized molecules after an 18-hour incubation with *Streptococcus gordonii*. Since bacteria uptake was used in this study, these experiments demonstrate a role for dendritic cell maturation in stabilization of class I molecules loaded with exogenous antigen (Rescigno et al., 1998). It is also known that the proteosome subunits, MECL-1, LMP2, LMP7, PA28α and PA28β are up regulated during DCs maturation. These changes in the proteosome could result in the generation of different peptides upon antigen processing (Macagno et al., 1999; Macagno et al., 2001).

A recent paper from Delamarre *et al.*, demonstrated that LPS-activated DCs increase their surface MHC I by 7-fold with in 30 hours. This group went on to show that, unlike MHC II complexes, MHC I complexes are not sequestered in immature DCs. Pulse chase experiments demonstrated a 2.6 fold increase in heavy chain synthesis in mature DCs when compared to immature DCs. The same 2.6 fold increase was observed for heavy chain-β2m complexes when measured by immunoprecipitation. After 15 minutes, the assembly of the complexes was only slightly more efficient in mature DCs

than in immature DCs. Although the kinetics of complex transport to the cell surface was nearly equal in the two cell types, MHC I complexes were slightly less stable on the surface of immature DCs. These data indicate that the observed higher levels of MHC I on maturing DCs is due to new protein synthesis and not assembly and recruitment (Delamarre et al., 2003)

Control Of Cross-Presentation During DC Maturation

Recent evidence suggests that the ability of DCs to cross-present exogenous antigen on Class I is also developmentally regulated. Using ovalbumin (OVA)-pulsed, LPS-stimulated DCs Delamarre *et al.*, demonstrated that cross-presentation of OVA occurs through the classical TAP-dependent pathway. To further support the idea that OVA gains entry to the cytoplasm in these experiments, these authors used FITC conjugated to OVA. Using immunofluorescent microscopy, they were able to demonstrate the presence of FITC-OVA in the cytoplasm within 30 minutes of phagocytosis, but before maturation was complete. The presence of OVA in cytosolic fractions was also confirmed by Western blot analysis. This group also demonstrated that although immature DCs could form the MHC I-OVA complexes, they could not cross-present antigen to $CD8^+$ transgenic OT-I T cells (specific for the immunodominant OVA epitope SIINFEKL) suggesting there are addition steps necessary for cross-presentation to be possible. In addition, only DCs matured through cluster disruption and LPS stimulation or CD40 ligation were able to cross-present OVA to the T cells, thus dendritic cell maturation is necessary, but not sufficient for cross-presentation to occur. By looking at the time course of cross-presentation ability, these authors showed that,

unlike endogenously synthesized antigens destined for class I presentation, antigen destined for cross-presentation are sequestered (possibly in the endoplasmic reticulum) in the immature DCs. By sequestering antigens in this manner, access to the proteosome is denied till full dendritic cell maturation occurs. Taken together these data suggest that the signals necessary for classical MHC I presentation, MHC II presentation and cross-presentation are all developmentally regulated, but are still distinct (Cella et al., 1997b).

In a more recent study, Gil-Torregrosa *et al.* investigated the effect of dendritic cell maturation on cross-presentation that was mediated by Fcγ expression by bone marrow-derived DCs. They found that the ability to cross-present antigen by these cells was possible during a very narrow window of development. Using LPS stimulation and immune complexed OVA antigen, it was shown that only intermediate DCs being able to cross-present antigen to the $CD8^+$ restricted T cells. These authors further demonstrated that intermediate cells were able to internalize antigen better than immature or mature DCs. LPS stimulation led to additional changes in the class I pathway of these DCs. Changes in proteosome subunits composition as well as changes in the rate of TAP-translocation were also observed in maturing DCs. Since introduction of antigen directly into the cytosol corrected the cross-presentation deficiencies in mature and immature DCs it was suggested that another post-endocytic event regulated by maturation must be involved in cross-presentation (Gil-Torregrosa et al., 2004).

Changes In MHC II Antigen Processing And Presentation in Mature Dendritic Cells

In addition to the normal intracellular endocytosis and exocytosis machinery professional APCs, such as DCs, contain specialized compartments for MHC Class II antigen processing and presentation. Immature DCs contain many vacuoles expressing late endosome/lysosomal markers (LAMP, lgp, hydrolases and low pH), which contain class II molecules and H-2M. H-2M's role in MHC II loading is to aid in the exchange of CLIP for higher affinity peptides. Once antigen is acquired, the DC changes it phenotype to that of an intermediate DC. In intermediate DCs, the MIIC compartments are replaced by the nonlysosomal vacuoles, CIIV, which now localize to the microtubule center (Mellman et al., 1998). The CIIV compartment no longer expresses lysosomal markers. The maturation of DCs also involves a series of intracellular changes involving MHC II molecules. The vacuoles containing the loaded MHC II molecules move to the surface of the DC and are displayed on the plasma membrane (Mellman et al., 1998). These changes in class II trafficking are thought to result in a type of antigenic memory such that the DC will be able to present the antigens it encountered as an immature cell. These changes also result in changes in trafficking that lead to waves of high level expression of MHC II-peptide complexes on the surface of the DCs, allowing the DCs to better stimulate $CD4^+$ T cells.

The changes in MHC II trafficking are also accompanied by biochemical changes in the stability of class II molecules. In immature DCs, class II molecules are unstable with a half-life of ~ 12 hrs before they are targeted to the lysosomes and degraded. This

degradation is independent of peptide loading. On the other hand, class II complexes in mature DCs have a half-life of >50 hours and are targeted from trans-golgi network to the plasma membrane (Mellman et al., 1998). Another maturation induced biochemical change may involve regulation of the cysteine protease Cathepsin S (cat S) by an antiprotease cystatin c. Cat S plays a role in the cleavage of the invariant chain during MHC II assembly. Cystatin c's role is to attenuate the protease activity of cat S and slow down invariant chain processing, resulting in the transport of the class II molecules to the lysosomes. In immature DCs there is enough cystatin present to perform this inhibitory function. Upon DC maturation, the levels of cystatin are decreased, thus increasing the active levels of cat S in the mature cells. This increase in cat S activity, results in the newly formed class II molecules avoiding the lysosomal degradation and thereby reaching the plasma membrane (Mawhorter et al., 1994). Recently, El-Sukkari *et al.* have knocked out cystatin in mice. The resulting characterization of the DC phenotype in these cystatin-deficient mice has shown no effect on the expression, sub-cellular localization, formation of peptide-loaded class II complexes or the efficiency of exogenous antigen processing (El-Sukkari et al., 2003).

The Role Of DCs In The Induction And Maintenance Of Peripheral Tolerance

Although $CD8^+$ and $CD8^-$ DCs are able to present antigen and activate T cells to enter into cell cycle, it has also been shown that $CD8^+$ DCs are less efficient at priming allogenic $CD4^+$ and $CD8^+$ T cells. In the case of $CD8^+$ T cells, the T cells stimulated by

$CD8^+$ DCs do not make enough IL-2 to sustain their proliferation and become anergic (Kronin et al., 1996), whereas $CD4^+$ T cells primed by $CD8^+$ DCs die by a Fas/FasL mechanism (Suss and Shortman, 1996). This led to the hypothesis that $CD8^+$ DCs may be the cells responsible for maintenance of self-tolerance. Belz *et al.* have shown that $CD8^+$ $CD11b^-$ DCs are involved in the induction of self-tolerance to tissue-associated antigen. Using mice transgenic for yellow fluorescent protein and a herpes simplex protein $gB_{498-505}$, these investigators were able to show by antibody depletion studies that only the $CD8^+$ DCs were able to induce production of β-galactosidase in a hybridoma cell line specific for herpes virus $gB_{498-505}$ and therefore were responsible for the tolerization of the antigen specific T cells (Belz et al., 2002a). On the other hand, this same group recently demonstrated the importance of $CD8^+$ dendritic population in the priming of CTL immunity to subcutaneous herpes virus infection as well as other viruses (Belz et al., 2004). Others have shown that the population of DCs responsible for presenting soluble antigen to T cells depends on the type of T cells. Using soluble OVA, Pooley *et al.* demonstrated that $CD8^-$ DCs are more efficient at presenting OVA to $CD4^+$ T cells whereas $CD8^+$ DCs are better at presenting soluble antigen to $CD8^+$ T cells (Pooley et al., 2001). In agreement with Beltz *et al.*, this group concludes that $CD8^+$ DCs are critical in cross-presentation (Belz et al., 2002b; Pooley et al., 2001). Due to the confusion in precisely typing DC, whether the $CD8\alpha^+$ DC involved in initiating the antiviral responses is the same as the $CD8\alpha^+$ population involved in tolerance induction is yet to be determined. It is possible that further subsets of this very heterogeneous population of

cells are yet to be elucidated. It is also possible that the inflammatory environment determines whether a DC is immunogenic or toleragenic and not the DC's lineage.

Others have proposed that the maturation state of the DC is what determines whether T cells are activated or tolerized. There are two ways in which T cell interactions with immature DCs could lead to tolerance induction. One possibility is that immature DCs will present low levels of peptide-MHC complexes without a costimulatory signal to the encountered T cells thus resulting in the T cells becoming anergic. Another situation that might result in tolerance is that intermediately mature DCs present antigen in the absence of inflammatory cytokines (Lutz and Schuler, 2002; Wilson and O'Neill, 2003). Other groups have shown that immunization with peptide-pulsed immature DCs inhibits effector T cell function (Dhodapkar et al., 2001). This same group went on to show that the peptide-pulsed immature DCs led to the induction of antigen specific $CD8^+$ regulatory T cells (Dhodapkar and Steinman, 2002). Regulatory T cells have been shown to be important in autoimmunity and tolerance. It has also been shown that DC maturation is important for cross tolerization of $CD8^+$ T cells (Albert et al., 2001). But like all things with this heterogeneous population of cells, maturation state alone may not be all that dictates which way the immunity versus tolerance scale is tipped. Menges *et al.* have demonstrated that repeated injections of TNF-α-matured DCs can lead to antigen specific protection of mice from recurring EAE (experimental autoimmune encephalomyelitis (Menges et al., 2002).

Macrophages

MΦs also arise from a bone marrow precursor and can be grown *in vitro* by placing BM progenitors in macrophage colony stimulating factor (M-CSF). MΦs exist in various forms in virtually every tissue in the body including secondary lymph organs like lymph nodes and spleen (Morrissette et al., 1999). Monocytes leave the bone marrow and enter the tissues via the blood stream. In some tissues, MΦs differentiate into specialized cells such as Kupffer cells in the liver or microglia cells in the brain. To become fully functional MΦs need to receive maturation signals such as cytokine tumor necrosis factor (TNF-α), LPS or interferon –γ. MΦs, like DCs, express MHC I, MHC Class II, CD11b, CD80 and CD86. Upon activation by inflammatory cytokines, CD40/CD40L interactions, B7/CD28 interactions or Toll receptors, the levels of all of these molecules are up regulated. MΦs are also able to migrate into the T cell area in the lymph nodes. In addition to all of these features, MΦs are highly phagocytic making them a good cell type to acquire foreign antigens and present them to T cells.

In vitro Evidence For A Role For Macrophages In The Initiation T cell Responses

Much evidence has accumulated supporting a role for MΦs in stimulation of T cell responses *in vitro*. Studies designed to determine the APC involved in presenting chemically modified allergens to $CD4^+$ T cells, showed that both MΦs and DCs, but not B cells were able to present these antigens to $CD4^+$ T cells clones isolated from allergic

patients. These studies also showed that both MΦs and DCs could restimulate Th0, Th1 and Th2 clones (Kahlert et al., 2000).

Since many human viral pathogens have gene products that can interfere with antigen processing and presentation, thereby allowing the virus to escape detection by the immune system, it may be critical to have numerous cell types capable of priming T cell responses. One such virus, cytomegalovirus (CMV), a β herpes virus, infects many cell types *in vivo* including MΦs and DCs. The priming of $CD8^+$ T cells and the generation of cytotoxic T cells are important for CMV clearance. CMV encodes two proteins that interfere with class I expression. One of these proteins causes it to be retained in the ER-golgi and the other protein binds and sequesters β2-microglobulin. It has recently been shown that these immune invasion genes are unable to down regulate class I in infected MΦs cell lines as well as in primary MΦs, allowing these cell to present class I restricted epitopes to CTLs. The authors conclude that MΦs play a critical role in the priming and maintenance of high precursor frequency in CMV (Hengel et al., 2000).

In vitro Evidence For A Role For Macrophages In Cross-presentation

As previously discussed, one characteristic that distinguishes professional antigen presenting cells from the non-professionals is the ability to cross-present soluble antigen on MHC I molecules to $CD8^+$ restricted T cells. Abundant literature exists on the ability of MΦs to present exogenous antigen to $CD8^+$ T cells (Brunt et al., 1990; Kapsenberg et al., 1986; Kovacsovics-Bankowski et al., 1993; Rock et al., 1993; Weinberg and Unanue, 1981). Using *Listeria*, Brunt *et al.*, demonstrated that MΦs infected with the intracellular

bacteria could present antigen to $CD8^+$ T cells. The ability of MΦs to cross-present bacterial antigens was shown to be dependent on the growth of the bacteria and expression of hemolysin by the bacteria *Listeria monocytogenes* (Brunt et al., 1990). In studies using OVA-specific T cell hybridomas, Rock *et al.* demonstrated that splenic and peritoneal MΦs can cross-present soluble ovalbumin to T cells hybridomas. Although the ability of DCs to cross-present exogenous antigen in association with class I could not be ruled out by these experiments, a role for B cells could be (Rock et al., 1993). In a later paper, this group also demonstrated that peritoneal MΦs and MΦ clones could also cross-present antigens (Kovacsovics-Bankowski and Rock, 1994). In addition, Kapsenberg *et al.* demonstrated using OVA-specific hybridomas that although both DCs and MΦs could cross-present soluble antigen to B cell depleted splenocytes, only MΦs could cross-present insoluble antigens (Kapsenberg et al., 1986).

In vivo Evidence For A Macrophages Role In The Initiation Of Immune Responses

Early studies had demonstrated that immunization with MΦs led to the priming of naïve T cell responses (Askonas et al., 1968; Unanue and Askonas, 1968). However, in these experiments MΦs may not be directly priming T cells because it is now known that DCs can acquire and cross-present antigen from other cells.

The role of MΦs in acquiring and presenting antigen to T cells has been demonstrated in viral models. By sorting APCs by FACS from the lymph nodes and spleens of Sendai virus infected mice, Usherwood *et al.* demonstrated that both MΦs and

DCs, but not B cells were able to present antigen to Senadai specific $CD4^+$ and $CD8^+$ T cell hybridomas *in vitro* (Usherwood et al., 1999). Hamilton-Easton *et al.* obtained similar results in an influenza mouse model. MΦs and DCs from mice infected with influenza were able to present antigen to virus specific MHC I restricted T cell hybridomas (Hamilton-Easton and Eichelberger, 1995).

A number of studies have attempted to deplete MΦs *in vivo* to determine the effect of depletion on T cell priming. Using carageenin and silica to deplete animals of phagocytic cells, numerous groups demonstrated the necessity for MΦs in the initiation of T cell responses. Debrick *et al.* studied the ability of silica carageenin depleted animals to mount $CD4^+$ and $CD8^+$ responses against influenza virus. Although $CD4^+$ proliferative responses to live virus were unaffected by silica treatment, CTL responses to the virus were completely abolished. One caveat of these experiments is the use of silica to deplete MΦs because it is possible for the silica to damage the highly phagocytic immature DCs in the same animal. However in Debrick's study in similarly treated animals, injection of peritoneal MΦs was able to completely restore CTL responses in these animals. To rule out the possibility of a contaminating cell type being responsible for the rescued CTL responses in these animals, these authors demonstrated the ability of a MΦ cell line to restore CTL responses. These data strongly suggest MΦs are sufficient for the generation of CTL responses to influenza (Debrick et al., 1991).

In similar experiments designed to investigate the mechanism of adjuvant activity, Wu *et al.* also showed the dependence of CTL generation on the presence of functional MΦs. These authors showed that macrophage depletion by silica carageenin treatment

had little effect (not statistically significant) on $CD4^+$ T cell proliferative responses or antibody titers, but drastically reduced CTL activity in animals immunized with soluble OVA and QS-21 (an adjuvant). The major flaw in depletion experiments using silica and carageenin is the ability of these treatments to damage the highly phagocytic immature DCs population in the same animal. However these authors went on to demonstrate that cultured splenic MΦs, but not DCs could reconstitute the CTL responses to almost normal levels (Wu et al., 1994).

In the study discussed above using exogenous hepatitis B surface antigen (HBsAg) particles pulsed APCs, Bohm, *et al.* also demonstrated that BM-derived and peritoneal MΦs could induce hepatitis specific CTL. Although elimination of MΦs and not DCs still resulted in CTL priming, elimination of both cell types by treatment with carrageenan suppressed CTL generation. Additionally in the carrageenan treated animals, CTL activity could be restored by immunization with HBsAg pulsed BM-derived MΦs (Bohm et al., 1995).

Another way to delete these highly phagocytic cells is through the administration of toxic liposomes. In studies using liposomes made from dichloromethylene diphosphonate, which is toxic for MΦs, Ciavarra *et al.* demonstrated that splenic red pulp MΦs are important for the initiation of both $CD4^+$ and $CD8^+$ T cell immunity against vesticular stomatitis virus. Interestingly, these authors also demonstrated that the toxic liposomes had no effect the ability of splenic APCs to stimulate mixed lymphocyte responses, suggesting the liposomes were not toxic to splenic DCs. These experiments suggest MΦs were sufficient to prime the VSV response (Ciavarra et al., 1997). Taken

together the silica/carageenin and toxic liposome experiments suggest that phagocytic cells are critical for the initiation of CTL responses, but the true identity of the antigen presenting cells in these studies still has not been elucidated.

Priming Of Naive T Cells

The predominant idea in the literature is that professional bone marrow-derived APCs are needed for the initiation of immune responses (Lenz et al., 2000; Sigal et al., 1999; Sigal and Rock, 2000). Cells of non-hematopoietic origin are thought to be unable to prime naïve T cell responses, because they lack the necessary costimulatory molecules and are unable to traffic to lymphoid organs. The need for the ability of a cell to migrate into the lymph node being necessary for a cell to function as an APC is supported by the fact that fibroblasts can initiate immune responses when delivered interperitoneally or directly into the spleen (Kundig et al., 1995). Kundig *et al.* interpreted these data to mean that a good APC doesn't need costimulatory molecules or the ability to secrete cytokines, but only the ability to home to lymphoid organs where T cells congregate. Using various model systems, this group ruled out the role of cross-presentation on the observed results (Kundig et al., 1995). A novel cell termed fibrocyte found in humans and mice, a fibroblast-like cell that are collagen^{+}/CD13^{+}/CD34^{+}/CD45^{+}, that can present antigen to naive T cells *in vitro* and *in vivo*. These cells have been shown to home to the site of a wound in response to CCR7 and are thought to play a role in wound healing and tissue repair (Abe et al., 2001). These cells also express class II, CD80, CD86 and adhesion molecules (CD11a and CD54) that are needed for antigen presentation. In addition fibrocytes were able to home to the draining lymph node when injected

subcutaneously. It has also been shown that HIV gp120 pulsed mouse fibrocytes were able to prime naive $CD4^+$ T cell *in vivo* (Chesney et al., 1997). Since these cells have class II on their surface, they will probably turn out to be a professional APCs. If these other cell types play a role in priming naive T cells *in vivo*, their role is a minor one. The bone marrow chimera data presented in this thesis as well as other studies in the literature underscore the importance and necessity of bone marrow- derived APCs in the initiation of immune responses (Lenz et al., 2000; Sigal et al., 1999; Sigal and Rock, 2000).

Costimulaton And Naive T Cell Priming

Efficient T cell priming requires interactions between the T cell and the antigen-presenting cell. The first interaction, signal 1, is the interaction of the T cell receptor with the peptide –MHC complex on the antigen-presenting cell. The second interaction (signal 2) involves the interaction of B7 on the APC with its receptor CD28 present on the T cell. The costimulatory signal is required for the optimal induction of IL-2 as well as the induction of the expression of the high affinity receptor for IL-2. The two signal hypothesis has been used to explain the need for both peptide-MHC/TCR interactions and costimulatory interaction for the initiation of naive T cell responses (Janeway, 1989; Lafferty and Woolnough, 1977; Mueller et al., 1989). Evidence exists that many molecules can serve to deliver the costimulatory signal to naive T cells. The list of molecules able to act as the costimulatory signal includes, but is not limited to CD40, OX40, LFA-1, ICAM-1, 4BB1, B7.1 and B7.2.

B7-CD28 interactions

The best-characterized costimulatory interaction involves B7's interaction with its receptor CD28. Over the last 10 years evidence has accumulated for the B7/CD28 interaction being the critical costimulatory interaction (Boussiotis et al., 1996; Van Gool et al., 1996). It has also been shown that TCR engagement the absence of B7 or CD28 signal (either by blockade or genetic knockout) leads to T cell anergy or nonresponsiveness (Boussiotis et al., 1996; Boussiotis et al., 1994; Guinan et al., 1994).

A major difference in the priming of naïve $CD4^+$ T cells versus naive $CD8^+$ T cells seems to be in the dependence on the need for costimulation at least in the response to viral pathogens. Using lymphocytic choriomeningitis virus (LCMV) and mice mutant in one costimulatory molecule as a model, it has been shown that the $CD8^+$ T cell response is still induced in CD40L, OX-40 and CD28 deficient mice. In this same model, the ability of $CD4^+$ T cells to mount an antiviral response was severely diminished. When 41BB knockout mice were infected with LCMV, the opposite pattern was observed, in that $CD8^+$ T cell responses were mildly compromised and $CD4^+$ T cells responses were unaffected (Seder and Ahmed, 2003; Whitmire and Ahmed, 2000). Others have demonstrated a need for costimulation in the generation of CTL responses to other viruses. Using vaccinia virus and exogenous ovalbumin as a model, Sigal *et al.* demonstrated a critical role for B7/CD28 costimulation is the generation of $CD8^+$ T cell responses (Sigal et al., 1998). Part of the role of costimulation may be able to be overcome by high dose antigen at least for $CD8^+$ alloresponses. Cai *et al.* demonstrated that although low level antigen stimulation required costimulation for the proliferative

response, high level antigen did not. These authors also showed that costimulation plays a role in lengthening the response by increasing IL-2 secretion (Cai and Sprent, 1996). In contrast, costimulation of $CD4^+$ with ICAM-1 in the absence of B7-CD28 interactions leads to functional anergy of the naive T cells (Ragazzo et al., 2001). These authors also demonstrated that stimulation of DO11.10 $CD4^+$ T cells with APCs that expressed ICAM-1 as well as B7.2 led to a decreased secretion of IL-4 by the proliferating T cells (Ragazzo et al., 2001).

Molecules other than B7 and ICAM-1, such as CD40, OX-40, 41BB, or LFA-1 can also serve as the 2^{nd} signal for T cell activation (Damle et al., 1992; Evans et al., 2000; Seder and Ahmed, 2003; Shinde et al., 1996; Whitmire and Ahmed, 2000). An important role for CD40-CD40L in initiation of both T and B cell responses has been demonstrated. Although, CD40L is not essential for T cell responses if the APC expresses costimulatory molecules, it is necessary for T cells to induce the expression of costimulatory molecules. It was also demonstrated that CD40 ligation led to the up-regulation of ICAM-1 and that proliferation of T cells in CD28 deficient mice is more dependent on ICAM-1 (Shinde et al., 1996). Naive B cells when used as antigen presenters usually lead to the tolerization of T cells. CD40 ligation on B cells leads to the up-regulation of the B7s and ICAMs, leading to the conversion of the B cell to a competent APC (Evans et al., 2000).

The Role Of Costimulation In Boosting Responses and in the Stimulation of Memory T Cells

Although the majority of evidence has demonstrated an important role for the B7/CD28 or B7/CTLA-4 interaction as being an important signal of the priming of T cells, there is evidence for other molecules serving the costimulatory signal. Using mice deficient in CD28 or mice deficient in heat stable antigen (HSA), Liu, *et al.* demonstrated a critical role for CD28 in the priming of naive effector CTLs in an influenza model (Liu et al., 1997). Using mice 8 days after influenza infection and taking advantage of the availability of antibodies against B7.1 and B7.2, this group further demonstrated in the HSA knockout animal, a requirement for either HSA or B7.1/B7.2 in the priming of a recall response. When these experiments were repeated, using mice 100 days after priming, similar results were obtained demonstrating that although HSA or CD28 can provide the necessary costimulation for memory induction, the B7-CD28 costimulatory signal is necessary for the induction of effector function from the memory T cells (Liu et al., 1997).

Effector T cells

As previously described naïve T cells need to receive a good quality signal from the APC. Three things contribute to this quality signal: the concentration of peptide-MHC complexes, the levels of costimulatory molecules and the duration of APC-T cell cognation (Lanzavecchia and Sallusto, 2002). It has been shown in $CD4^+$ T cells that there are different levels of TCR signaling needed for naïve $CD4^+$ T cells to acquire

different effector functions. A low strength signal will induce proliferation and the ability to home to the lymph nodes, but not effector function. A medium strength signaling will induce and cytokine exposure will stimulate Th1 or Th2 effector differentiation and the T cells gain the expression of adhesion molecules necessary for migration into peripheral tissues. Really high levels of antigenic stimulation will lead to T cell death via AICD. This hierarchy of antigenic signal determining T cell fate has been termed the progressive differentiation model by Lanzavecchia (Lanzavecchia and Sallusto, 2002). Others have shown that even a limited exposure to antigen causes both $CD4^+$ and $CD8^+$ T cells to undergo an antigen independent program of proliferation and differentiation (Masopust et al., 2004; Seder and Ahmed, 2003; van Stipdonk et al., 2003; van Stipdonk et al., 2001).

The commitment of naïve T cells to the proliferation program can occur in as little as 6 hours if there is high dose antigen and high level stimulation (Masopust et al., 2004). On the other hand, the length of stimulation needed to commit naïve T cells to the proliferation program for low dose antigen and low level costimulation can be as long as 40 hours (Lanzavecchia and Sallusto, 2002). After a naïve T cell encounters antigen it will proliferate and differentiate into an effector cell. Once activated, the T cell will start a new transcriptional program that will express genes involved in cell cycle, cytokines and cytokine receptor genes, adhesion molecule genes involved in homing and finally genes involved sensitivity to activation induced cell death (AICD) (Lanzavecchia and Sallusto, 2002). Both naïve $CD4^+$ and $CD8^+$ express next to no mRNA for the various effector molecules they will need to express. The expression of these molecules involves

a lot of signaling molecules and intracellular events. The effect of this priming and the steps needed for a T cell to differentiate into a effector and then on to a memory T cell, are different for the $CD4^+$ and $CD8^+$ subsets (Masopust et al., 2004; Seder and Ahmed, 2003).

$CD8^+$ T cells require a very short time for antigen exposure to become committed to rapidly enter division and undergo a very rapid proliferation program (6-8 divisions a day) (Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2003; van Stipdonk et al., 2001). $CD4^+$ cells do not have this dynamic programming and require prolonged antigen exposure to proliferate (Foulds et al., 2002). $CD8^+$ T cells are also able to develop into effector cells faster than $CD4^+$ T cells. This has been demonstrated using adoptive transfer of ovalbumin (OVA) specific $CD4^+$ and $CD8^+$ T cells into wild type mice that were then infected with a recombinant *Listeria monocytogenes*, an intracellular bacterium, which expresses OVA. In this model 8 days after infection >85% of $CD8^+$ T cells had become effectors as determined by IFN-γ staining, whereas only ~7% of $CD4^+$ T cells stained for the effector cytokine (Foulds et al., 2002). This ability of $CD8^+$ T cells to expand significantly better than $CD4^+$ T cells holds true in other viral models as well, including Sendai (Cauley et al., 2002), LCMV (Homann et al., 2001) and vaccinia virus (Harrington et al., 2002) infection in mice and Epstein-Barr virus infection in humans (Maini et al., 2000). The same trend has been shown for the predominance of $CD8^+$ T cells in the response to *Listeria*. However, there are other models of infection where more $CD4^+$ T cells go on to become IFN-γ secreting effector cells i.e. *Leishmania major* (Belkaid et al., 2002; Mendez et al., 2001) and *Mycobacterium tuberculosis* (Lima

et al., 2001; Silva et al., 1999; Winslow et al., 2003). It is interesting to note that these differences could also be explained by differences in the ability of the various antigens to be presented as well as the ability of the pathogen to directly infect professional APCs.

$CD8^+$ Effector T Cells

Once stimulated the effector molecules produced by $CD4^+$ and $CD8^+$ T cells differ. In the case of $CD8^+$ T cells the major effector cytokines are TNF-α and IFN-γ. Cytotoxic T cells (CTL) recognize abnormal cells by detecting antigenic peptides bound to the target's MHC class I molecules. In addition to those two cytokines, $CD8^+$ CTL also produce molecules needed to induce apoptosis in their targets. Once the target cell is recognized, the CTLs will kill them by FasL induced ligation of Fas or the secretion of perforin and granzyme B (Henkart, 1994; Henkart and Sitkovsky, 1994). The peptides displayed on MHC class I molecules of most cells are generated from the genes being expressed within that cell. Consequently, infected cells will display peptides from intracellular pathogens and cancerous cells will present peptides from abnormally expressed genes. Effector CTLs circulate looking for cells expressing such foreign peptides and eliminate them. CTL can also be broken down into subsets. There are Tc1 and Tc2 $CD8^+$ T cells. Tc1 cells express the chemokine receptor CCR5 and Tc2 cells express the chemokine receptor CCR4. Both subsets of $CD8^+$ cells have cytolytic capability. The cytokine environment can also effect $CD8^+$ T cells development. Cytokines like IL-2 and IL-15 promote $CD8^+$ T cell differentiation (Lanzavecchia and Sallusto, 2002). There is also evidence for a role in inhibition of T cell responses by cytokines, such as transforming growth factor β.

$CD4^+$ Effector T Cells

$CD4^+$ T cells are generated by the presentation of antigenic peptides on MHC class II molecules. The main function of $CD4^+$ T cells is the secretion of various helper cytokines that aid in the development of the various other immune cells. Effector $CD4^+$ T cells can be broken down further into 3 subsets Th0, Th1 and Th2. Th0 cells are thought to be the least differentiated and express predominantly interleukin-2 (IL-2), and granulocyte macrophage colony stimulating factor (GM-CSF). Th1 cells predominantly produce IFN-γ. IFN-γ activates MΦs to produce various cytokines as well as generate oxygen intermediates important for their microbicidal function. Th1 cells can also secrete IL-2, TNF-α and lymphotoxin. These cytokines play a key role in the elimination of intracellular pathogens, MΦ activation and delayed type hypersensitivity responses. Th2 T cells secrete interleukin-4 (IL-4), IL-5, IL-9, IL-10 and IL-13. These cytokines are important for B cell activation and isotype switching as well as allergic responses. It has also been recently shown that many genes are differentially expressed by the subsets. These include surface markers as well as chemokines. Th1 cells preferentially express IFN-γ receptor- β chain, interleukin-12 receptor β chain, interleukin-18 receptor, P-selectin glycoprotein-1, CXCR3 and CCR5. Where as Th2 cells preferentially express the IL-1-like molecule T1/ST2, CCR3, CCR4 and CCR8. This difference in the ability to produce the various cytokines is regulated at the level of transcription factors. Th1 cells express the transcription factor T-bet, where as Th2 cells express the transcription factor gata-3 and c-Maf (Glimcher and Murphy, 2000). Cytokines also have an important role in both $CD4^+$ T cell subset differentiation. IL-4 stimulation of Th0 cells induces the

expression of the transcription factors Gata-3 and c-Maf, which in turn induces Th2 specific genes in $CD4^+$ T cells. On the other hand, if the $CD4^+$ T cell is exposed to IL-12 it will express the transcription factor t-bet and develop into a Th1 type cell (Glimcher and Murphy, 2000; Lanzavecchia and Sallusto, 2002). There is also evidence that high TCR signal strength of TCR can cause up regulation of T-bet and GATA 3, suggesting that skewing of $CD4^+$ T cell differentiation, can occur in the absence of cytokines. It is known that Il-12 is important in the stimulation of Th0 cells to become Th1 cells and that IL-4 is important for the differentiation of Th0 cells into Th2 cells.

What other factors contribute to the polarization of $CD4^+$ T cells into the two subsets is still an area of very active investigation. Recent evidence has demonstrated a role for the stimulating APC. Some groups have shown that $CD8\alpha^+$ DCs produce IL-12 and preferentially stimulate $CD4^+$ T cells to become Th1 cells (Glimcher and Murphy, 2000). It has also been demonstrated that myeloid DCs lead to the development of Th2 cells possibly by the secretion of IL-6 (Diehl and Rincon, 2002). There has also been data suggesting a role for antigen dose and structure. Experiments involving the use of altered peptide ligands have demonstrated a role for strength of TCR signal in determining lineage commitment (Glimcher and Murphy, 2000).

Whether an animal responds with a Th1 or Th2 slanted response has been shown to be important in various disease models. In the case of intracellular parasites like *Leishmania donovani,* a Th1 response is needed for clearance. The $CD4^+$ cells secrete interferon-γ, which activates the bactericidal mechanisms of MΦs to clear the parasite. In the case of helminth infections like *Schistosoma mansoni*, Th2 responses are correlated

with worm clearance. This is because antibodies play an important role in the resolution of helminth infections.

What factors play roles in Th1 versus Th2 $CD4^+$ T cell development *in vivo* are still an unknown. One factor that may play a role is the nature of the priming APC. In experiments using MΦs cell lines loaded *ex vivo* with soluble hemagglutinin, Desmedt *et al.* showed that MΦs were indeed able to prime $CD4^+$ T cell responses. These authors further demonstrated that this priming was not dependent on host APCs by repeating the experiments in H-2 mismatched mice. These authors went on to demonstrate that the majority of $CD4^+$ T cells primed in these mice were Th1 type cells by ELISPOT analysis. This skewing of the $CD4^+$ T cell differentiation was further supported by the exclusive generation of IgG2a and IgG2b in these mice (Desmedt et al., 1998). Other studies have also shown differences in $CD4^+$ T cell populations when various APC are used as stimulators (Croft et al., 1992.; Duncan and Swain, 1994)

Other $CD4^+$ T cells can differentiate into suppressor or regulatory T cells. Regulatory T cells can be further broken down in Tr1 and Th3 cells. $CD4^+CD25^+$ Tr cells are important in the maintenance of tolerance and in suppressing immune responses. $CD4^+CD25^+$ Tr cells arise in the thymus and are self- antigen reactive, where as Tr1 and Th3 cells arise in the periphery after antigen stimulation. These cells are known to secrete IL-10 transforming growth factor-β (TGFβ) and IL-4. Tr1 cells secrete IL-10 with or without TGF-β, IL-5 or IL-13. These cells make little or no Il-2, IL-4 or IFN-γ. Th3 cells secrete high levels of TGF (Mills and McGuirk, 2004).

Activation Induced Cell Death

Due to the possibility of tissue damage inflicted by renegade T cells, the overall expansion of effector T cells is regulated by many redundant mechanisms. After activation, the expanded T cells will undergo a contraction, where 90% of the effectors will be killed and the surviving T cells will give rise to the memory pool. It is also known that during viral infections the $CD8^+$ T cells undergo a more drastic contraction phase than the $CD4^+$ T cells (Seder and Ahmed, 2003). It has been demonstrated during viral infections that the magnitude of the T cell response at the peak of the response will predict the size of the memory pool (Seder and Ahmed, 2003). Some of the molecules thought to be involved in this process (termed activation induced cell death or AICD) are Fas/FasL, TNF and its 2 receptors, IFN-γ, CTLA-4 and CD40/CD40L (Lenardo et al., 1999; Seder and Ahmed, 2003; Whitmire and Ahmed, 2000; Whitmire et al., 2000). There has also been a role demonstrated for the withdrawal of IL-2 (Blattman et al., 2003; Seder and Ahmed, 2003). $CD4^+$ and $CD8^+$ T cells are susceptible to these mechanisms to varying degrees. The effect of various death molecules on $CD4^+$ versus $CD8^+$ T cell homeostasis can be most clearly seen in the Fas-deficient mice, where the lymphoproliferation of T cells is predominately in the $CD4^+$ compartment (Hildeman et al., 2002). In addition, it has been shown $CD8^+$ T cells are more sensitive to the death signals transduced through the TNF receptors than are the $CD4^+$ T cells (Speiser et al., 1996; Sytwu et al., 1996; Zheng et al., 1995). Recently a role for perforin and IFN-γ has been shown for the regulation of effector $CD8^+$ T cells homeostasis (Badovinac et al., 2000; Kagi et al., 1999; Sad et al., 1996; Zarozinski et al., 2000; Zhou et al., 2002).

Memory T cells

The ability of an organism to generate an effective and stable memory pool may determine its ability to fight off a secondary infection by the same organism. It also has become evident in recent years that a host's repertoire of memory cells and previous infection history can highly influence the immune response to a new pathogen (Selin and Welsh, 2004). The question of how memory T cells arise is still an open one. Requisite characteristic of memory T cells are the ability to stably persist, to rapidly respond to antigen and the ability to self renew (Wherry et al., 2003). Currently there are 3 models proposed by Wherry: the linear model, the decreasing potential model and the progressive differentiation model. The linear model says that the effector T cells that survive the contraction phase are destined to become memory T cells. The decreasing potential model says that once the antigen experienced T cells enter division, one of the daughter cells form the early division is to become the mother of the memory population and that the requirements for the generation of effector and memory function may be different (Liu et al., 1997). The third model put forth by Lanzavecchia *et al.* proposes a role for the strength and duration of TCR signaling. This model is termed the progressive differentiation model (Lanzavecchia and Sallusto, 2002). Presently the linear model for memory generation is the most favored model.

There has been a significant amount of data published in the last five years supporting the linear differentiation model for memory generation (Opferman et al., 1999:Wherry, 2003 #434; Wherry et al., 2003)]. Using transgenic T cells specific for the male H-Y antigen and stimulated with peptide *in vitro*, Opferman *et al.* showed that it

took 5 divisions for the H-Y T cells to gain effector CTL function. By transferring "pre-effector" T cells and "post-effector" T cells into congenic naive mice, this group was able to demonstrate that only the "post-effector" T cells gave rise to memory cells. They concluded that although effector CTL are susceptible to AICD, it is this population of cells that gives rise to long-term memory cells (Opferman et al., 1999). Using lymphocytic choriomeningitis virus (LCMV) and the intracellular pathogen, L*isteria monocytogenes*, Wherry *et al.* also demonstrated that memory is linear (Wherry et al., 2003).

The literature has shown that only a few things about memory cells can be generalized because this is a highly heterogeneous population of cells. It has also been suggested that the generation of memory cells may be different for the different T cell subsets. Recently there has been a general role demonstrated in the expression of the chemokines CCR7 as distinguishing between 2 different types of memory that is consistent between $CD8^+$ and $CD4^+$ T cells effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). CCR7 mediates the ability of T cells to home to the lymph nodes via the high endothelial venules (Seder and Ahmed, 2003; Wherry et al., 2003)]. It has been demonstrated that $CCR7^-$ T_{EM} cells found in the nonlymphoid tissues, spleen and blood are faster at making effector molecules; where as $CCR7^+$ T_{EM} cells found in the lymphoid tissues are slower at gaining effector functions (Seder and Ahmed, 2003; Wherry et al., 2003). Both populations of memory cells have been shown to be capable to eliciting recall responses (Lanzavecchia and Sallusto, 2002; Wherry et al., 2003). The question as to whether central memory and effector memory arise from a common

precursor or different stages of differentiation for the same cell remains unresolved. Data from Wherry *et al.* using LCMV has suggested that effector memory cells give rise to central memory cell (Wherry et al., 2003). In contrasting reports, Lanzevecchia's group proposes that central memory cells give rise to effector memory cells (Geginat et al., 2003a; Geginat et al., 2003b). And finally, Baron *et al.* suggests that human effector and central memory cells may be separate population unable to interconvert (Belz et al., 2004). These discrepancies in the literature may be due to the differences between T cell populations i.e. $CD4^+$versus $CD8^+$ T cells. Another possibility is that the "rules" are not the same for viral antigens as they are for antigens generated from intracellular parasites. It is also important to note that most of these studies were done using replicating viruses or intracellular parasites, which would result in high antigenic burdens.

Many papers over the last 5 years have demonstrated that the generation and persistence of memory cells is not dependent on the presence of antigen. These experiments have been done by immunizing mice, waiting for them to reach the memory state than transferring them into MHC knockout mice (Murali-Krishna et al., 1999; Swain et al., 1999). This is in sharp contrast to naive $CD4^+$ and $CD8^+$ T cells, which require MHC interactions to survive (Ernst et al., 1999; Kirberg et al., 1997; Kirberg et al., 2001; Takeda et al., 1996). A role for IL-7 and IL-15 in the maintenance of memory has been established for $CD8^+$ T cells (Marrack et al., 2000; Sprent and Surh, 2001). Although MHC class I is not needed for the survival of memory $CD8^+$ T cells, it is necessary for the adequate expansion and attainment of effector function upon challenge (Kassiotis et al., 2002).

Rationale

The predominant view in the literature today is that only DCs efficiently prime naive T cells responses at least in certain situations (Banchereau and Steinman, 1998; Guermonprez et al., 2002; Jung et al., 2002; Norbury et al., 2001; Steinman et al., 1983; Steinman and Witmer, 1978). Interestingly, in some studies DCs are the only antigen-bearing APCs isolated from antigen injected animals (Jung et al., 2002; Norbury et al., 2002). Early studies have suggested that MΦs could stimulate primary immune responses, but the current view is that MΦs play a role in immune responses as effector cells not as the APC initiating the responses.

In vitro there is abundant evidence that MΦs can acquire antigen and present it to $CD4^+$ T cells (Askonas et al., 1968; Hsieh et al., 1993a; Hsieh et al., 1993b; Kahlert et al., 2000; Unanue, 1984; Unanue and Askonas, 1968) and $CD8^+$ T cells (Hamilton-Easton and Eichelberger, 1995; Usherwood et al., 1999) . Moreover, it is also well established that, *in vitro*, MΦs can take up and present particulate and cell-associated antigens on MHC class I and class II molecules (Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1994; Nair et al., 1995). Similarly, MΦs have been isolated from animals injected with viruses (Hamilton-Easton and Eichelberger, 1995; Usherwood et al., 1999) or soluble antigen (Grant and Rock, 1992) and shown to present antigen to T cells *ex vivo*. It is also well established that MΦs acquire antigens from pathogens *in vivo* and present them to T cells (Neild and Roy, 2003; Ziegler, 1984). This is basis for host defense against many intracellular pathogens and type IV hypersensitivity responses (Janeway et al., 1999). Therefore, MΦs clearly acquire antigens *in vivo* and generate

peptide MHC complexes. In addition, MΦs in peripheral tissues that have acquired foreign material migrate to regional lymph nodes (Kotani et al., 1979; Shi and Rock, 2002). MΦs that reside in the lymph nodes and spleen also perform a filtering function and capture antigen present in blood or lymph (Maino and Joris, 2004). In all of these situations MΦs are stimulating previously activated (effector) T cells or hybridomas (Janeway et al., 1999).

Naïve T lymphocytes have more stringent requirements for activation than effector T cells and hybridomas. The issue of what APC initially stimulates these cells to initiate immune responses is an important one. It is well established that DC can stimulate primary immune responses. It is often argued that DCs are the only APC that initiate primary immune responses and that MΦs play no role in this process (Banchereau and Steinman, 1998; Inaba and Steinman, 1986; Mellman and Steinman, 2001; Mellman et al., 1998; Norbury et al., 2002; Steinman et al., 1983; Steinman and Witmer, 1978). However, the published evidence supporting this view is relatively limited. Much of the evidence comes from *in vitro* experiments. However, it is unclear whether these *in vitro* experiments accurately model what occurs *in vivo*.

In summary, DCs can stimulate primary T cell responses and may be particularly potent in doing so. However, on close examination the evidence that they are the only cells that can prime responses and that MΦs lack this capability is relatively scant. It is possible that DCs are the principal APCs for presenting some antigens (e.g. listeria and malaria). Whether MΦs are also sufficient to prime naïve $CD4^+$ T cell responses remains to be determined.

The ability of both MΦs and DCs to acquire and present antigens, express costimulatory molecules and migrate to the lymph nodes led us to hypothesize that both MΦs and DCs would directly activate naïve $CD8^+$ T cells and that this priming would lead to functional effector cells as well as functional memory T cells.

Chapter II: Materials and Methods

Animals

C57BL/6, B6/SJL F1, Rag $^{-/-}$, BALB/c, β2-microglobulin deficient (B6.129P2-B2m^{tm1Unc}), mice deficient in Fas (B6.MRL.TNFrsf6 ^{lpr}) Perforin knockouts (C57BL/6-Pfp^{tm1Sdz}) and mice congenic for Ly 5.1/CD45.1 (B6.SJL-Ptprc^{a} Pep3^{b}/ Boy) and Thy 1.1 /CD90.1 (C57BL/6-Igh^{a} thy1^{a} Gpi1^{a}) were initially obtained from The Jackson Laboratories (Bar Harbor, Maine) and /or bred in specific pathogen-free conditions at UMASS Medical School. T cell receptor transgenic (TCR) mice OT-I (specific for the ovalbumin peptide SIINFEKL in the context of K^{b}) were obtained from Steve Jameson (Univ. of Minn). DO11.10 transgenic mice transgenic mice specific for the 323-339 peptide of ovalbumin in the contact of I-A^{d} were the kind gift of Dr. Dale Greiner (UMASS Medical School). Breeding pairs of the P-14 mice specific for KAVYNFATC peptide from LCMV gp33 in the context of D^{b} were the kind gift of Dr. Ray Welsh (UMASS Medical School). C57BL/6 mice transgenic for green fluorescent protein were the gift of Dr. Rachel Gerstein (UMASS Medical School). All T cell receptor transgenic mice were bred as heterozygotes and housed in the UMASS pathogen free animal facility. The transgenic colonies were maintained by breeding transgenic mice to wild type or congenic C57BL/6 mice. LCMV and OT-I mice were typed using Vα2 Vβ5.1 antibodies for OT-Is and Vα2 Vβ 8.1 antibodies for P-14 mice and FACS analysis. DO11.10 mice were typed by staining with the clonotypic antibody anti-KJ1–26 and analyzed by FACS.

Antibodies And Reagents

All antibodies for FACS analysis unless noted were purchased from Pharmingen/ BD (San Diego, CA). The clonotypic antibody anti-KJ1–26 was purchased from Caltag (Burlingame, CA). Anti-CD45.1 was purchased from ebioscience (San Diego, CA). Intracellular cytokine reagents including antibodies were also purchased from Pharmingen/ BD (San Diego, CA). Peptides with the following sequences were generated by Steve Reed at Corixa (Seattle, WA): ISQAVHAAHAEINEAGR (DO11.10), SIINFEKL (OT-I) and KAVYNAFATC (gp33)

Generation of F1 chimeras

B6/SJL F1 mice were lethally irradiated using 1200 rad. Bone marrow was prepared from the femurs and tibias of C57BL/6 mice or B10.S-$H2^s$/sgMcdJ donor mice and depleted of T cells using a monoclonal antibody against Thy 1, M5/149 (ATCC) and complement (PEL-Freeze). The irradiated mice were reconstituted with $2.5x10^6$ T cell depleted-bone marrow cells given intravenously. The mice were than housed for 3-4 months in a SPF environment to allow for the turnover and reconstitution of the antigen presenting cells in the peripheral tissues. The reconstitution of the chimeras was tested by adoptively transferring transgenic T cells (either OT-I or P-14) and challenging the animals with OVA coated beads (Kovacsovics-Bankowski et al., 1993) or a 13mer from gp33, KAVYNFATCGIFA (Ciupitu et al., 1998), both of which have previously been shown to prime naïve T cells in a D^b restricted manner. In all cases, these antigens failed to stimulate the TCR-tg T cells in F1 chimeric animals whose bone marrow lacked H-2^b, indicating that the host APCs had been completely eliminated.

Production of bone marrow-derived macrophages and dendritic cells

Femurs and tibias were collected from C57BL/6 mice and flushed using RPMI-1640 containing 10% FCS, 2 mM glutamine, 1X antibiotic-antimycotitc, 100 mM HEPES, 100 μM nonessential amino acids and 5 x 10^5 2-mercaptoethanol, complete media (Life Technologies, Carlsbad, California). The red blood cells were lysed using ACK Red Cell lysis buffer and the cells were washed. The cells were resuspended in complete RPMI and plated in non-tissue culture treated Petri dishes for an overnight incubation at 37 °C in 5% CO_2 incubator. The next day, nonadherent cells were collected, counted and adjusted to 0.5 x10^6 cells/ml in complete media. BM cells (7.5 x10^6 cells) were plated in 100 mm tissue culture suspension dishes (Costar) in the presence of 10 ng/ml GM-CSF and 5 ng/ml IL-4. BM cells were plated in the same manner except DAP supernatants were added as a source of M-CSF at a final concentration of 10%. DAP supernatant were generated by culturing confluent DAP cells for 5-7 days. Supernatants were collected and filter sterilized and stored at –80^0C. The BM cells were incubated at 37 °C in 5% CO_2 incubator. After 7 days, the cells were removed from the dishes using rubber policemen. The APCs were incubated with the indicated peptides for 2 hours in complete RPMI and then washed 3 times with Hanks Balanced Salt Solution (HBSS) without Mg^{+2} and Ca^{+2} (Gibco).

Analysis of peptide-MHC complexes

MΦs and DCs, incubated with 10 μM SIINFEKL in complete RPMI at 37 ° C in a 5% CO_2 incubator for 4 hours, were washed 3 times in HBSS and replated in complete RPMI at 0.5 x10^6 cells/ml. Cells were scraped up at various time points and incubated

with biotinylated 25D1.16 antibody for 45 minutes. 25D1.16 recognizes SIINFEKL in the context of K^b (Porgador et al., 1997). Samples were then washed 2 times with HBSS + 5% FCS and incubated with Strep-avidin-APC (BD/Pharmingen) for an additional 45 minutes. Samples were washed twice with HBSS+5% FCS. The cells were then fixed using 1 % paraformaldehyde and stored at 4° C until analysis. Samples were analyzed using a FACS Calibur and FlowJo analysis software.

Adoptive Transfer Experiments

Adoptive transfer experiments were done as previously described by Jenkins (Ingulli et al., 1997). Briefly, T cells were isolated from lymph nodes of transgenic mice and purified by nylon wool or complement depletion (Pel –Freeze) depletion using an anti-class II (M5/114) and an anti-HSA antibody, J11D, ATCC). OT-I/Rag $^{-/-}$, OT-I/GFP or P-14/Rag$^{-/-}$ animals were also used as a source of T cells for some experiments. The transgenic T cells are then labeled with 1 μM CFSE (Molecular Probes, Eugene, OR) in HBSS without serum for 20 minutes at 37 °C. The cells were than washed once with HBSS without Mg^{+2} and Ca^{+2} (Life Technologies) supplemented with 5% FCS followed by serum free HBSS. One day prior to immunization, $2.5x10^6$- $4.0\ x10^6$ transgenic T cells were adoptively transferred into host animals, which resulted in Tg T cells consisting of about 1% of $CD8^+$ T. On day 0, DCs or MΦs pulsed with the appropriate peptide in complete media for ~3 hours in 5% CO_2 at 37^0C. After washing 3 times without serum the $0.5x10^6$ or $1x10^6$ APCs were injected either subcutaneously or intravenously, depending on the experiment. Non peptide-pulsed APCs were used as a negative control.

FACS Staining and Analysis

Draining lymph nodes and/or spleens were harvested at various times after APC transfer and made into single cell suspensions. The cells were incubated in 2.4G2 to block Fc receptors for 10 minutes. The cells were then stained with PerCP conjugated anti-CD8 (Pharmingen) and either allophycocyanin conjugated anti-Ly 5.1(Pharmingen) or Cy 5 conjugated anti-Thy 1.1 (e Bioscience San Diego, CA)) and their CFSE content was examined using flow cytometry.

Antigen Presenting Cell Migration Experiments

Cells were removed from the Petri dishes and labeled with 1 μM Cell Tracker orange (excitation at 548 nm and emission at 576 nm) or Cell Tracker green (Molecular Probes) dye (excitation at 492 nm and emission at 517 nm) HBSS without serum at a concentration of $<25x10^6$ cells/ml for 45 minutes at 37° C. The cells were then washed once with 5% FCS in HBSS and 2 more times with HBSS without serum. Draining lymph nodes were harvested and incubated with 2.4 μg/ml collagenase and 1 μg/ml of DNase (Boehringer Mannheim) for 90 minutes at 37 ° C. The lymph nodes were then drawn through a 23 G needle to make a single cell suspension. The cells were then washed twice and allowed to recover for 2 hours at 4° C in complete media. The cells were stained with antibodies against Ly 5.1 (Pharmingen) and analyzed by FACS. In some experiments the cells were tracked by CFSE labeling before injection or GFP content.

Fluorescent Microscopy

The draining lymph nodes were removed at various times after DCs and snap frozen in "Tissue-Tek" OCT compound (Miles Inc.) on a dry ice ethanol bath. Sections were than made on a microtome at 4 μm or 25 μm thicknesses. The sections were mounted on glass slides and fixed in 4% paraformaldehyde for 20 minutes. 4 μm sections were examined using a Olympus fluorescent scope with a CAD camera mount. Analysis was performed using Adobe Photoshop. The 25μm sections were analyzed using the UMASS microscopy core facility and Z-axis deconvolution software.

Intracellular Cytokine Staining

Adoptive transfer experiments were done as described above. Three or four days after transfer of the APCs, lymph nodes and/or spleens were removed, minced and made into a single cell suspension. Cells were stimulated *in vitro* with either media alone or with the cognate peptide (SIINFEKL 1 μg/ml or KAVYNAFATC 5 μg/ml) in the presence of Brefeldin A for 5 hours. The samples were then washed and stained for $CD8^+$ and Ly5.1or Thy 1.1 depending on the experiment for 30 minutes. The samples were then fixed with CytoPerm/CytoFix (Pharmingen, San Diego, CA) for 20 minutes in the cold. The cells were then permeabilized with Perm/Wash (Pharmingen, San Diego, CA). PE-Anti-IFN-γ, clone XMG (Pharmingen, San Diego, CA) was added in the presence of Perm/Wash for 45 minutes. The samples were washed twice assayed by flow cytometry.

In Vivo Cytolysis

The *in vivo* cytolysis assays were done as described previously by Ahmed *et al.* (Barber et al., 2003). Briefly, at various times points after priming, peptide pulsed 5 μg/ml and unpulsed Ly 5.1 CFSE labeled splenocytes targets were injected intravenously. Twenty to twenty-four hours later, lymph nodes or spleens were removed and stained with APC- anti-Ly 5.1. Samples were analyzed by flow cytometry. Percent killing was calculated according to the following formula: 100-{[(%peptide pulsed in primed)/% unpulsed in primed)/% peptide pulsed in unprimed/% unpulsed in unprimed)] X 100}.

Plaque Assays

At various times after immunization with peptide coated APCs, mice were challenged with 4-5x10^4 PFU LCMV interperitoneally. Three days after infection, spleens were removed and homogenized using a tissue homogenizer. The homogenates were spun to remove debris and frozen at -80 °C. 10-fold serial dilutions of the homogenates were made and added to veros cell monolayers plated at ~60-70 % with an agar overlay. 24 hours after infection, neutral red was added in more overlay mixture. 24-48 hours later, plaques were visualized using a light box and counted. Viral titers were calculated according to the following formula: PFU/ml = plaques counted x dilution x volume added).

Chapter III: Macrophages Prime Naïve $CD8^+$ T Cells In Wild Type Mice

Results

Dendritic Cells And Macrophages Can Both Stimulate Primary T Cell Responses In Vivo

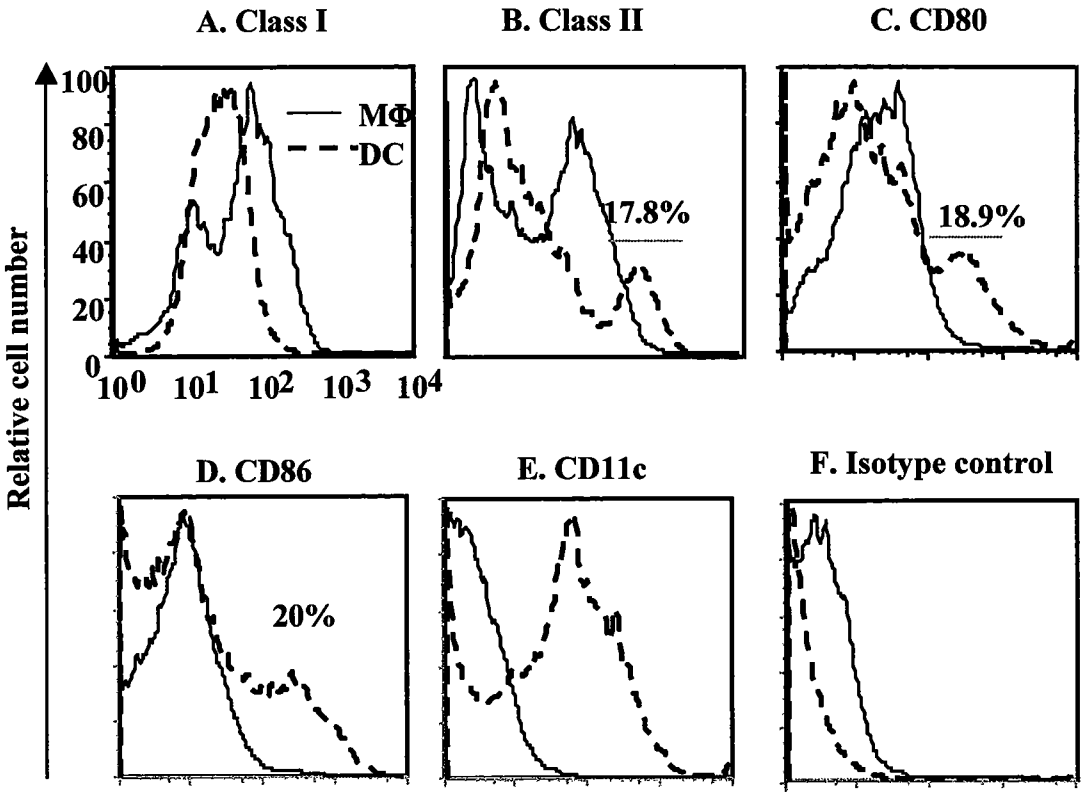
In order to study the ability of different types of antigen presenting cells to prime naïve T cells, we grew MΦs and DCs from mouse bone marrow. We first analyzed these cells for their expression of CD11c, MHC molecules, CD80, and CD86. Of the bone marrow cells cultured in GM-CSF and IL-4, about 70% differentiated into DCs, as defined by expression of the pan DC marker CD11c; although these cells were not homogeneous, we will henceforth refer to them as DCs. The DCs used in this thesis are the myeloid derived Class II^+ $CD11b^+$, $CD11C^+$ DCs. In contrast, the bone marrow cultured in M-CSF, which stimulates the growth of MΦs, totally lacked the expression of the CD11c DC marker (Fig. 1). The presence of these phenotypic markers in addition to the morphology of these cells led to the classification of them as MΦs.

Although the MΦs are biphasic for MHC class I expression, the majority of them express similar levels of these molecules as DCs. Similarly, the levels of CD80 and CD86 on the majority of both cell types are about the same. Although the majority of DCs (~80%) are of the immature phenotype as defined by surface markers, a small subpopulation of the DCs (~20%) is more mature as indicated by the higher expression of class II, CD80 and CD86. The population of MΦs was also biphasic for MHC class II

Figure 1: Characterization Of APC Populations.

Bone marrow-derived MΦs and DCs were cultured as described in the Material and Methods section of this thesis. In all panels MΦs are shown in the solid line and DCs are shown in the dashed line. Panel A shows the levels of MHC I, panel B= MHC II, panel C=CD80, panel D= CD86, panel E=CD11c and panel F is Pe-anti IA^d antibody used as a negative control. This experiment was repeated 4 times.

Figure 1: Characterization of APCs

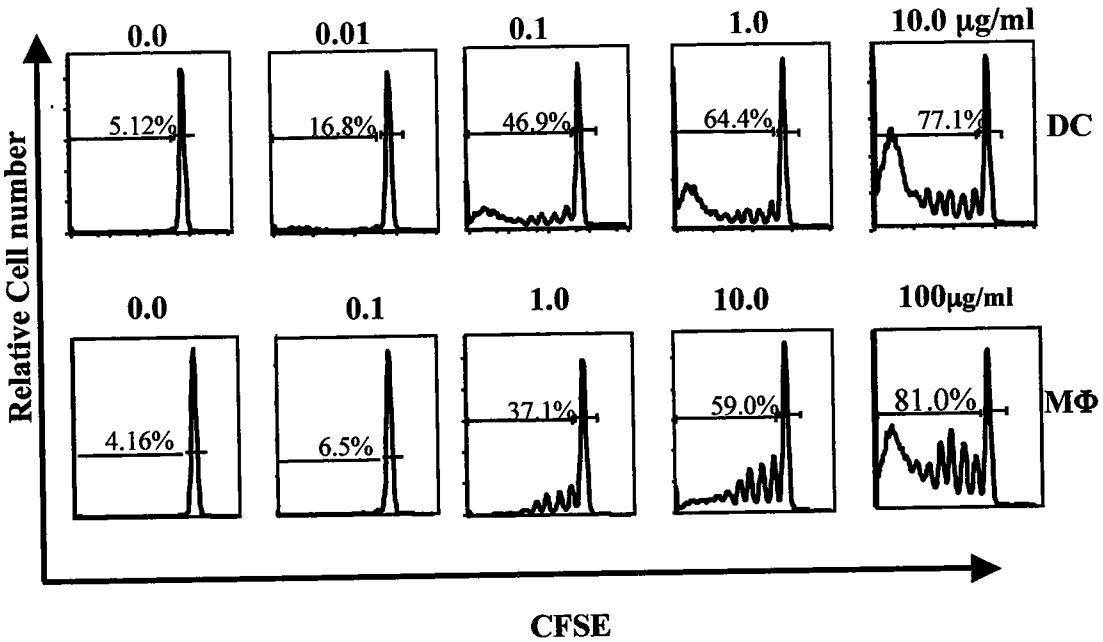


with about 40 % expressing moderate levels and 60% expressing low to undetectable levels of class II molecules (Fig. 1). To test the ability of these APCs to stimulate T cells, we used an adoptive transfer system similar to that described by Jenkins *et al.* $CD8^+$ T cells from P-14 LCMV T cell receptor transgenic mice, specific for the gp33 peptide (KAVYNFATC) presented on D^b (Pircher et al., 1993), were labeled with CFSE and transferred intravenously into C57BL/6 mice. Twenty-four hours later these mice were injected subcutaneously into the footpad with gp33-pulsed bone marrow-derived DCs or MΦs. Unpulsed APCs were used as a negative control. Four days after the injection of the APC, the draining lymph node (popliteal) was removed and made into a single cell suspension. Lymph node cells were stained for CD8 and CD45.1 or CD90.1 to identify the antigen specific T cells and analyzed by flow cytometry. In animals immunized with either MΦs or DCs pulsed with peptide, the transgenic T cells proliferated significantly, as indicated by the progressive dilution of CFSE label with each division (Fig. 2). In contrast, antigen specific T cells did not proliferate in animals primed with unpulsed DCs or MΦs. Figure 2 also demonstrates that the amount of peptide needed to induce proliferation of the antigen specific T cells is about 10-fold higher for MΦs than it is for DCs when they are injected subcutaneously. Thus it takes at least 1 μg/ml of KAVYNFATC to elicit a T cell response when MΦs are used as the priming APC where as only 100 ng/ml is needed for DCs. At the high (non-limiting) doses of KAVYNFATC DCs and MΦs stimulated equal proliferation and percentage accumulation of terminally divided T cells (77% for DCs vs. 81% for MΦs)(Fig. 2).

Figure 2: Titration Of Gp33 Needed To Prime Naive P-14 T Cells

Draining lymph nodes were harvested four days after APC transfer, minced, stained and analyzed by FACS analysis. The X- axis depicts the level of CFSE content of the transgenic T cells whereas the Y-axis represents relative cell number. The different panels represent the increasing concentration of gp33 used to pulse the APCs. The top row of panels shows the results of DC immunization with the various peptide concentrations and the bottom row shows the results for MΦ immunizations in the same experiment. This experiment was repeated at least five times with a total n=6 animals for the lower peptide concentrations and n>25 for the higher peptide concentrations.

Figure 2: Macrophages Prime Naïve P-14 T Cells



To make sure this stimulation of transgenic T cells was not peculiar to a particular TCR transgenic system, the experiment was repeated using different TCR transgenic T cells. We adoptively transferred OT-I T cells specific for the SIINFEKL peptide (OVA-p) present in the context of K^b (Hogquist et al., 1994) into C57BL/6 mice (Fig 3). The different color traces represent two different animals. We frequently see some level of animal-to-animal variation in these types of experiments. The MΦs were also able to stimulate naive OT-I T cells. A similar 10-fold difference in potency was observed when MΦs and DCs coated with SIINFEKL were used to stimulate ovalbumin-specific OT-I transgenic T cells (Fig 3).

The prevailing view has been that DCs are the principal cells that stimulate naïve T cells (Banchereau and Steinman, 1998; Belz et al., 2002c; Steinman et al., 1983). It was therefore surprising to find that MΦs stimulate such strong primary T cell responses. Consequently, we evaluated whether contaminating DCs might actually account for the ability of cultured MΦs to prime T cell responses. As described above, the cultured MΦs contained <1% (essentially undetectable) cells expressing the CD11c DC marker (Fig. 1), which is expected because DCs are not stimulated by the M-CSF used to grow MΦs. We tested whether 1% or less of contaminating DCs would be sufficient to prime T cells. As shown in Fig. 4, the minimum number of DCs needed to prime T cell responses in our system was ~ <50,000, which is far above what could be contaminating our MΦ cultures.

Figure 3: Macrophages Prime Naive OT-I T Cells

CD90.1 congenic lymph node cells OT-1 T cells were transferred IV into C57BL/6 mice. The next day, mice were immunized SQ with APCs pulsed with various concentrations of OVA-p peptide. Three days later, the draining lymph node was removed and stained with antibodies against CD8 and CD90.1. The samples were gated on live, $CD8^+$ and $CD90.1^+$ cells and analyzed for CFSE content. The red and blue lines represent independent mice from the same experiment. This experiment was repeated 3 times with a total n=8 mice/peptide concentration.

Figure 3: OT-I Dose Response to SIINFEKL Peptide

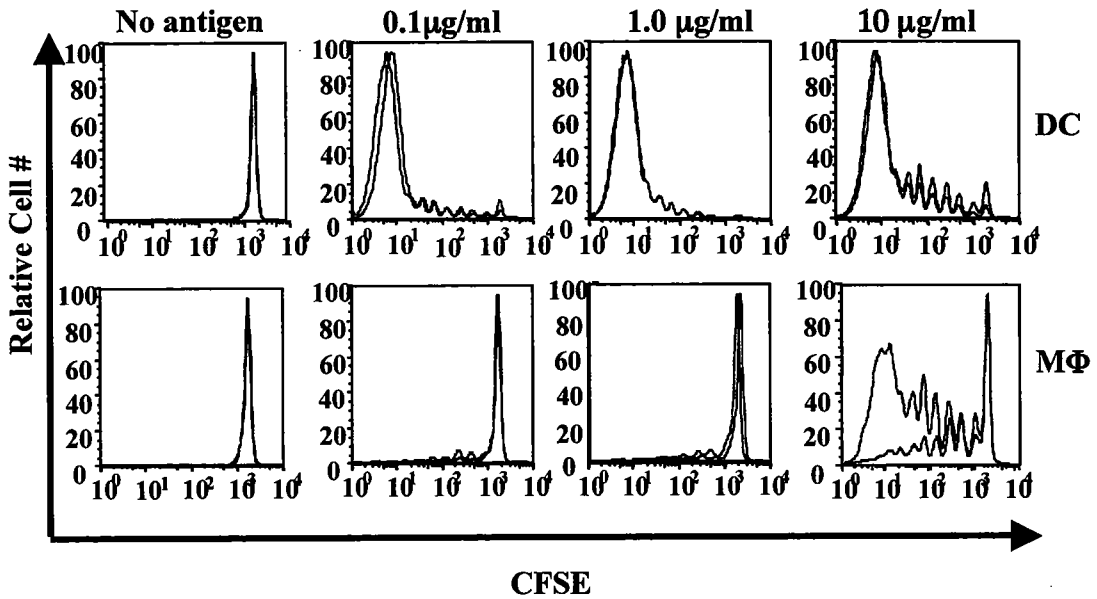
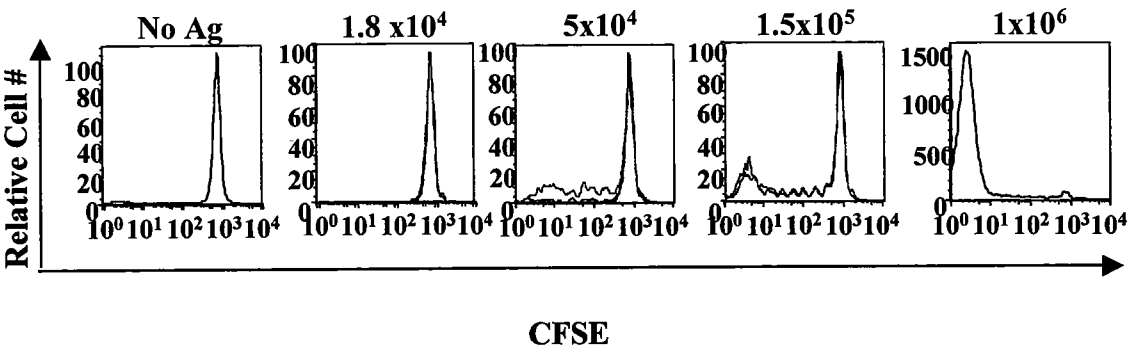


Figure 4: At Least 50,000 Dendritic Cells Are Needed To Elicit T Cell Responses.

$CD45.1^+$ congenic CFSE labeled P-14 T cells were adoptively transferred into C57BL/6 mice. The next day, mice were immunized IV with various numbers of DCs pulsed with 10 μg/ml of the gp33 peptide. Three days later, the draining lymph node was removed and stained with antibodies against CD8 and CD45.1. The samples were gated on live, $CD8^+$ and $CD45.1^+$ cells and analyzed for CFSE content. The different color lines represent independent mice from the same experiment. This experiment was repeated 3 times with a total n=9 mice/cell number.

Figure 4: Number of Dendritic Cells Needed to Induce T Cell Proliferation



To further assess this point, we also tested MΦ and DC lines, which are cloned and lack any contaminating cell type (Kovacsovics-Bankowski and Rock, 1994; Shen et al., 1997). We found that both the C2.3 and A3.1 MΦ cell lines stimulated primary OT-I T cell responses, and did so equivalently to the DC 2.4 cloned DC line (Fig. 5). In these panels the red trace represents the degree of T cell proliferation observed when unpulsed cells lines are used as an APC. We believe this moderate level of background is due to the presentation of endogenous peptides or serum proteins by the cloned cells. These experiments were repeated using P-14 T cells and the C2.3 MΦ cell line and DC2.4 DC line (Fig. 6) and again demonstrated MΦ and DC priming of T cells. In this figure the different lines represent the result of independent mice. As noted above, variability between animals is frequently observed. Overall, we conclude that peptide-pulsed MΦs can stimulate primary T cell responses and that these responses are not being stimulated by contaminating DCs.

Comparison Of Dendritic Cell And Macrophage Priming Of T Cell Responses.

As shown in the previous section, the degree of T cell proliferation as well as the amount of T cells present in the terminal division peak is dependent on the dose of peptide used to coat the APCs (Figure 2) as well as the cell type used for presentation. The overall level of proliferation and accumulation of terminally divided antigen specific T cells in the P-14 system was similar when animals were stimulated with either MΦs or DCs at high doses of peptide, but the kinetics of the responses were different.

Figure 5: Cell Lines Can Stimulate Naive OT-I T Cells.

OT-I lymph node T cells were adoptively transferred into C57BL/6 mice. The next day, mice were immunized SQ with the two MΦ cell lines and one DC line pulsed with various concentrations of SIINFEKL peptide. Four days later, the draining lymph node was removed and stained with an antibody against CD8. The samples were gated on live and $CD8^+$ cells and analyzed for CFSE content. Panel A shows the proliferation seen when MΦ cell lines are used as the stimulation APC. Panel B shows the results obtained when a DC cell line is used as the stimulating APC. The red line represents the background T cell proliferation seen with unpulsed cell lines used as a negative control. The blue line represents T cell proliferation in the mouse that received OVA-p pulsed APCs. This experiment was repeated 3 times with 2 mice being immunized with each concentration of peptide pulsed cell line for a total n=6 mice/group.

Figure 5A: Stimulation of OT-I T Cells by Macrophage Cell lines

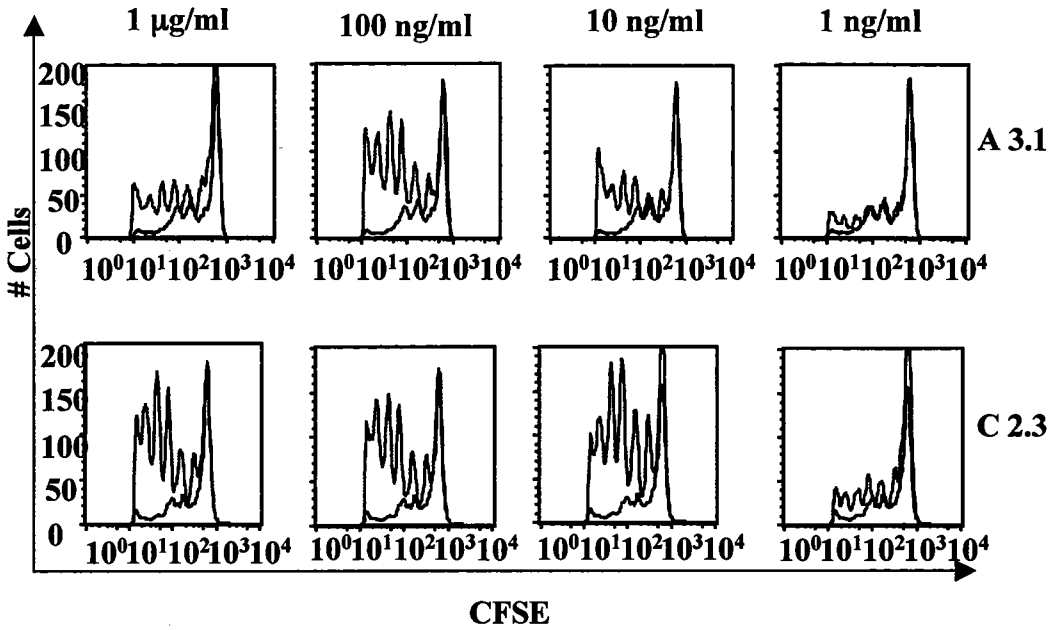


Figure 5B: Stimulation of OT-I T Cells by Dendritic Cell Line

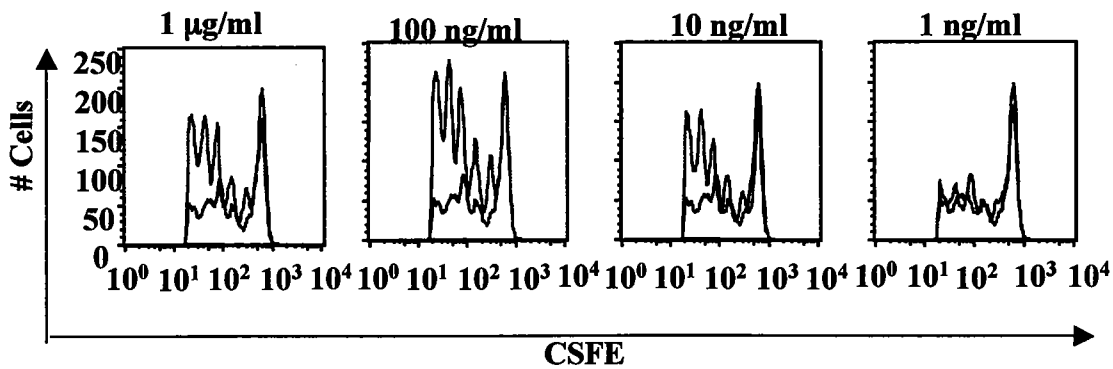
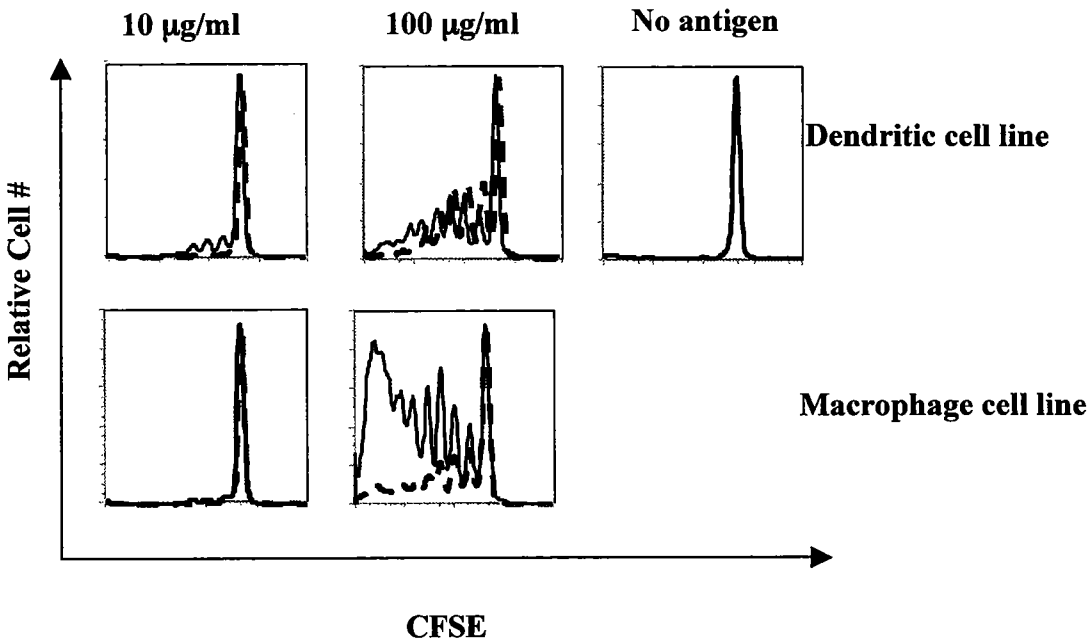


Figure 6: Stable Cell Lines Can Also Stimulate Naive P-14 T Cells.

CD90.1 congenic P-14 T cells isolated from lymph nodes were adoptively transferred into C57BL/6 mice. The next day, mice were immunized SQ with cell lines pulsed with various concentrations of gp33 peptide. Three days later, the draining lymph node was removed and stained with antibodies against CD8 and CD90.1. The samples were gated on live, $CD8^+$ and $CD90.1^+$ cells and analyzed for CFSE content. The black and blue lines represent two independent mice from the same experiment. This experiment was repeated 3 times with a total n=6 mice/peptide-pulsed cell line.

Figure 6: Stable Macrophage and Dendritic Cell Lines Prime Naïve P-14 T Cells



Transgenic T cells in the MΦ primed animals consistently reached their peak frequency 3 days after priming, whereas the peak was on day 4 in animals primed with DCs (Fig 7 A).

The ability of the MΦs and DCs to stimulate equal T cell expansion in the P-14 system was in contrast to what was seen when OT-I T cells are used as the responding T cells. When OT-I T cells were adoptively transferred and stimulated with peptide pulsed MΦs given subcutaneously, the peak accumulation was less than seen when the T cells were stimulated with DCs. This can be seen in Figure 7B. When transgenic T cells were primed with DCs, up to 38 % of the $CD8^+$ T cell pool were OVA-p specific. In contrast, transgenic T cells stimulated by peptide-pulsed MΦs only expanded to ~10 % of the $CD8^+$ T cell pool. The reason for the difference in the amount of expansion of OT-I versus P-14 T cells stimulated with MΦs is not known. Nevertheless, given that the initial precursor frequency of OT-I T cells was only about 1% the expansion seen with both APCs is still impressive. The experiment was also done using CD90.1 congenically marked OT-I T cells.

It is possible that the 10-fold difference in the concentration of peptide needed to elicit T cell responses by DCs versus MΦs was due to differences in their respective expression of costimulatory molecules, although the majority of these cells express similar levels of CD80 and CD86 after *in vitro* culture (Fig.1).

Figure 7: Overall Accumulation Of Antigen Specific T Cells.

A. $2.5x10^6$ $CD45.1^+$ P-14 T cells labeled with CFSE were transferred into wild type hosts. One day later the animals were immunized SC with either gp 33-pulsed MΦs (100 μg/ml) or gp33-pulsed DCs (10 μg/ml). The draining lymph nodes were removed 2, 3, 4, 5 and 7 days after APC transfer. Single cell suspensions were made and the cells were stained with CD45.1 and CD8 and analyzed by FACS. The red trace represents an animal that received gp33-pulsed DCs and the blue trace represents an animal that received gp33-pulsed MΦs. This experiment was repeated 3 times with a total number of a total n= 6 mice/time point.

B. $2.5x10^6$ GFP^+ OT-I transgenic T cells were transferred into C57BL/6 hosts. One day later the animals were immunized SC with MΦs or DCs pulsed with 1 mg/ml of OVA-p. Draining lymph nodes were removed 2, 3, 4, 5 and 6 days after APC transfer. The lymph nodes were made into single cell suspensions and stained with anti-CD8 antibodies and analyzed by FACS. The data are depicted as the percentage of $CD8^+$ T cells expressing GFP. The red (MΦ) and green (DC) traces represent animals that received unpulsed APCs. The fuchsia (DC) and blue (MΦ) traces represents animals immunized with APCs pulsed with OVA-p. This experiment was repeated 3 times with a total number of a total n= 6 mice/time point.

Figure 7A. Overall Accumulation of Antigen Specific P-14 T Cells

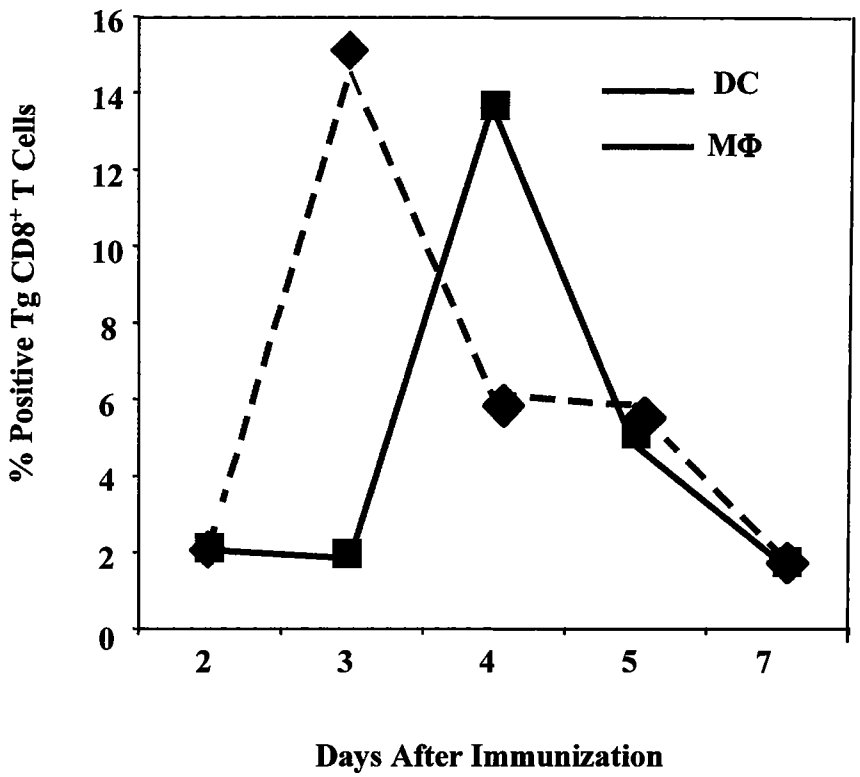
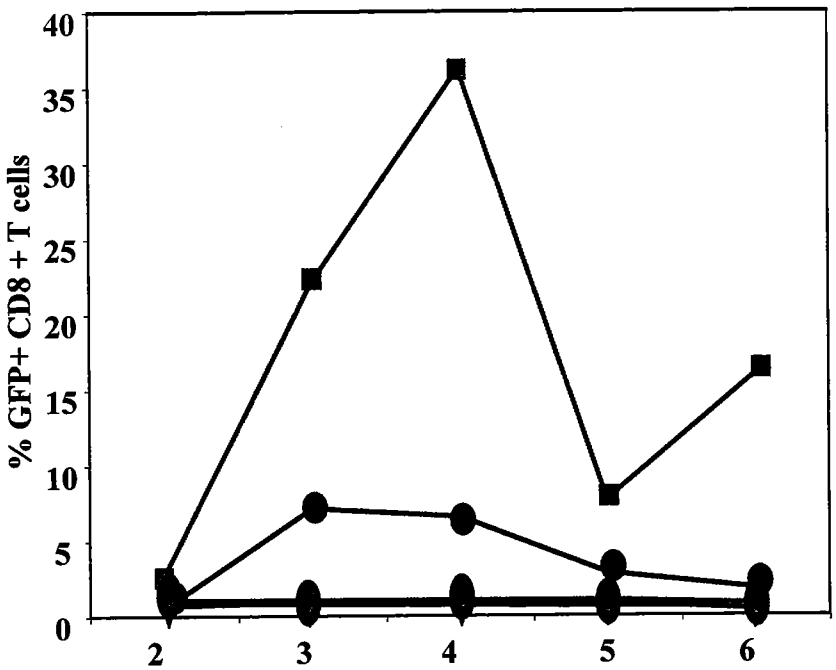


Figure 7B: Accumulation of OT-I Cells



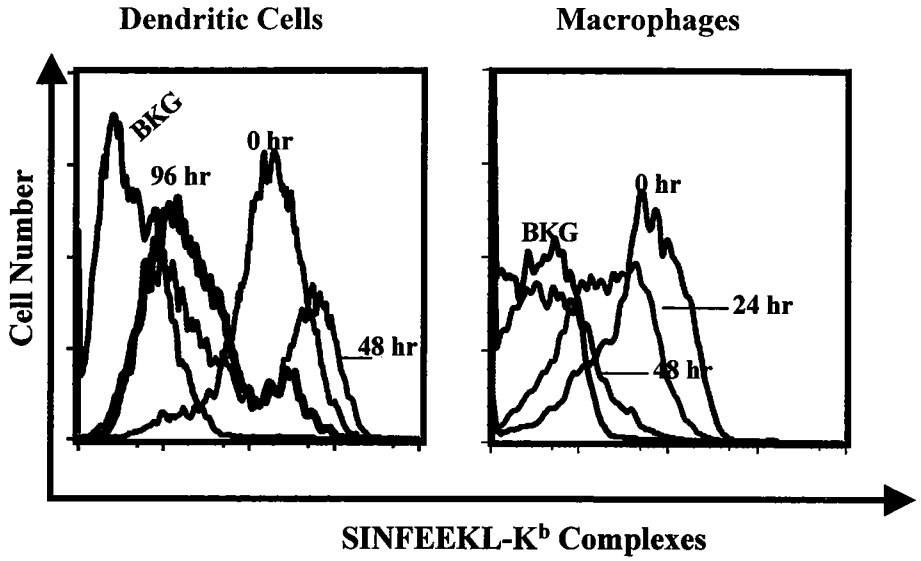
Other possible differences include the level of level of MHC I-peptide complexes and the ability of the APCs to home out of the subcutaneous space in to the lymph node. To investigate some of the other possible basis for this difference in potency we performed several additional experiments. We first examined whether the cells were presenting similar numbers of peptide-MHC complexes. For this analysis we used the ovalbumin peptide SIINFEKL because this system also shows a similar potency difference between MΦs and DCs and the number of K^b-SIINFEKL complexes can be quantified with the mAb 25D1.16. MΦs and DCs were incubated with SIINFEKL peptide, stained with 25D1 and analyzed by flow cytometry. Unpulsed APCs were used as negative controls. Similar numbers of K^b-SIINFEKL complexes formed on the peptide pulsed MΦs and DCs (Fig.8). Interestingly, however, we observed a vastly different half-life of the complexes on the two cell types. Even after 96 hours, the vast majority of DCs still expressed detectable levels of K^b-SIINFEKL and, remarkably, on a subpopulation of DCs the levels of these complexes was essentially unchanged after 4 days. The cells that still have high-level complexes at 96 hours are the CD80/CD86, class II high population of mature DCs (data not shown). In sharp contrast, MΦs have lost most if not all of their K^b-SIINFEKL complexes by 48 hours.

While the differences in the expression of costimulatory molecules and half-life of peptide-MHC complexes might contribute to the difference in potency between MΦs and DCs, we also examined whether DCs and MΦs migrate with

Figure 8: SIINFEKL-K^b Complexes Are More Stable On the Surface of Dendritic Cells than On Macrophages.

Bone marrow-derived MΦs and DCs were cultured as previously described. The APCs were than pulsed with 10 μg/ml of OVA-p for 4 hours. The cells were than washed extensively and replated. Cells were harvested at 0, 24, 48, 72 and 96 hours after pulsing and stained with biotin-labeled 25D1. After incubation with strepavidin-APC the cells were washed and analyzed by FACS. Panel A shows the half-life of SIINFEKL-K^b complexes on DCs and Panel B shows the same data for MΦs. The black line represents the background levels of 25D1 staining on unpulsed APCs. This experiment was repeated with 4 different DC and MΦ preparations.

Figure 8: Half-life of SIINFEKL-k^b Complexes on the Surface of the APCs

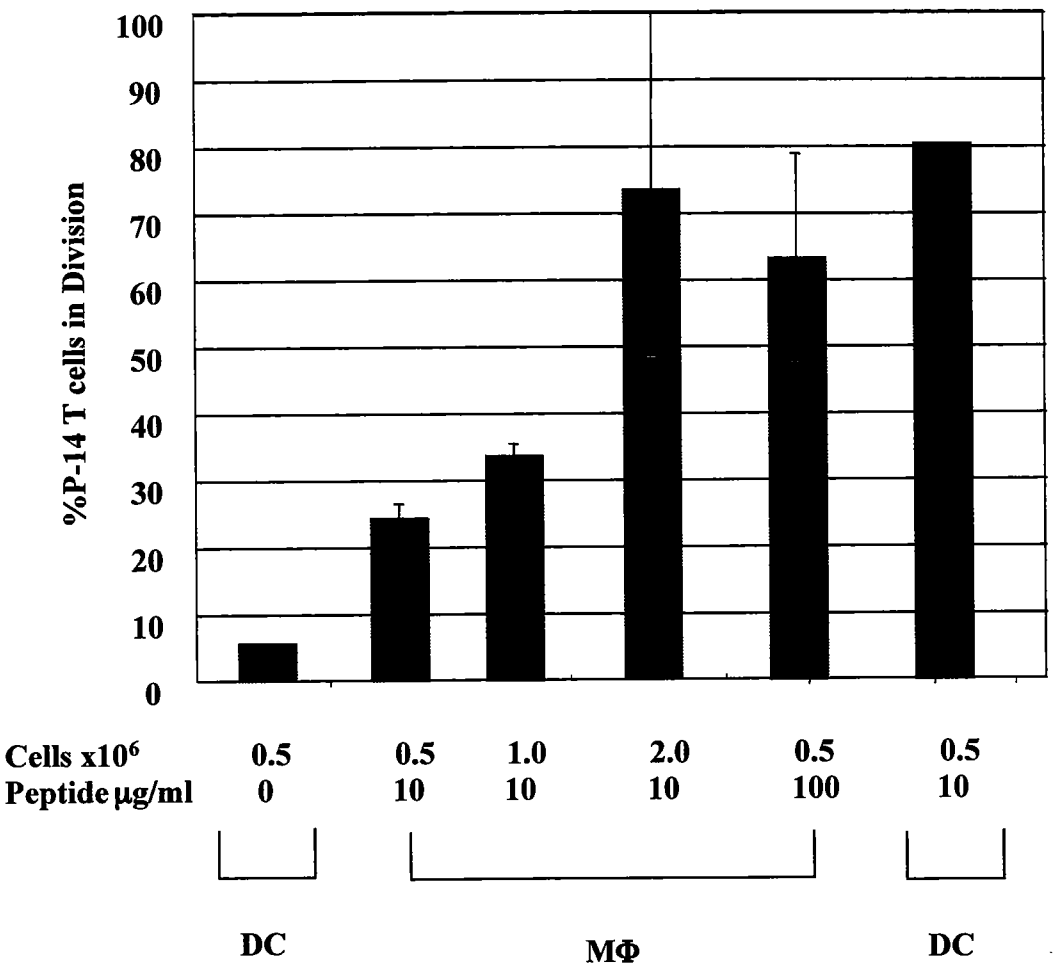


different efficiencies. In initial studies we injected mice subcutaneously with dye-labeled APCs and then examined sections of the draining lymph nodes. After 24 hours, we observed by fluorescent microscopy that there were more labeled DCs than labeled MΦs in the draining lymph nodes (data not shown). To determine whether the lesser migration of MΦs contribute to their reduced potency relative to DCs in priming T cells, we performed two additional experiments. The number of APCs that migrate to the lymph node is proportional to the number injected. Therefore, in the first experiment, we injected mice with four times the number of peptide-pulsed MΦs, while keeping the number of DCs constant. Under these conditions, MΦ and DCs stimulated an equal expansion of the antigen specific T cells to the same concentration of peptide. Increasing the number of MΦs injected had a greater effect than increasing the peptide concentration 10-fold (Figure 9). In a second experiment, MΦs and DCs were pulsed with 10 μM of gp33 and injected intravenously one day after the transfer of CFSE labeled P-14 transgenic T cells into C57BL/6 mice. We reasoned that the intravenous injection of MΦs and DCs might allow the two APCs to home in more similar numbers to the spleen and lymph nodes.

Figure 9: Injecting More Macrophages Result is Better T Cell Priming.

$2.5x10^6$ $CD90.1^+$ P-14 T cells labeled with CFSE were transferred into wild type hosts. One day later the animals were immunized with 0.5 $x10^6$ (turquoise), 1 $x10^6$ (dark blue) or 2 $x10^6$ (bright green) MΦs pulsed with 10 μg/ml of gp33. Additional animals were immunized with 0.5 $x10^6$ MΦs pulsed with 100 μg/ml of gp33 (fuchsia). 0.5 $x10^6$ unpulsed DCs (black) or DCs pulsed with 10 μg/ml of gp33 (red) were used as a positive and a negative control, respectively. Lymph nodes were harvested on day 3 from the animals immunized with MΦs and on day 4 from the animals immunized with DCs because this is the day of peak T cell proliferation (see Fig. 7 A). The data are represented as percentage of $CD8^+$ T cells that are transgenic T cells. This experiment was repeated 3 times for a total of 6 mice/group.

Figure 9: Injecting More MΦ Results in More T cell Accumulation



To analyze the proliferation of the P-14 transgenic T cells after intravenous injection of the APCs, inguinal lymph nodes and spleens were removed 3, 4 and 6 days post injection. The results of a representative experiment are shown in Figure 10. By day 2, one can detect the presence of transgenic T cells that have proliferated in the spleens of animals primed with both MΦs and DCs. Proliferating transgenic T cells continued to be present in the spleens of these animals from day 3 through day 6. The lymph nodes of these animals present a slightly different picture. Three days after immunization, only mice injected intravenously with peptide-pulsed MΦs have proliferating T cells in the draining node. From day 4 through day 6, essentially equal numbers of proliferating transgenic T cells can be detected in the lymph nodes of animals immunized with DCs and MΦs. Therefore, immunization of animals with 10μg/ml of gp33-pulsed APCs via the intravenous route led to identical levels of P-14 transgenic T cell proliferation and expansion (Fig.10). In case we were missing the difference in the ability of the different APCs to prime via the IV route, we repeated the experiments using APCs pulsed with a series of 3-fold dilutions of gp33. This experiment was done as previously described. Three days after APC transfer, the mice were sacrificed and spleens were removed. The results of a representative experiment can be seen in Fig. 10 B. The result of this experiment was identical to those of the experiment described above. This set of experiments demonstrates that when MΦs are given intravenously they are able to prime naïve P-14 transgenic T cells as effectively as DCs.

Figure 10: Macrophages Delivered IV Prime As Well as Dendritic Cells.

A. $2.5x10^6$ CFSE labeled $CD90.1^+$ P-14 T cells were adoptively transferred into C57BL/6 mice. The next day, the animals were immunized with MΦs or DCs pulsed with 10μg/ml of gp33 via the intravenously route. After various days, spleens and inguinal lymph nodes and spleens were removed, stained for CD8 and CD90.1 and analyzed by FACS. Animals that received MΦs are shown by red lines and animals that received DCs are shown by black lines. This experiment was repeated 4 times with a total n=10 mice/group.

B. $2.5x10^6$ CFSE labeled $CD90.1^+$ P-14 T cells were adoptively transferred into C57BL/6 mice. The next day, the animals were immunized with MΦs or DCs pulsed with 0.1, 0.3, 1.0 and 10μg/ml of gp33 via the intravenously route. Three days later, the spleens were removed, stained for CD8 and CD90.1 and analyzed by FACS. The different color lines represent 2 or 3 animals in the same treatment group. This experiment was repeated 2 times with a total n=6 mice/group.

Figure 10A: MΦs Prime Naïve T Cells As Well As DCs When Injected Intravenously

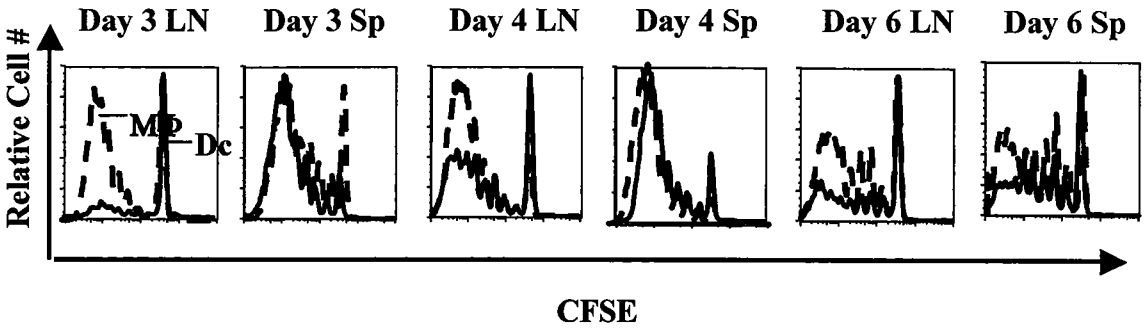
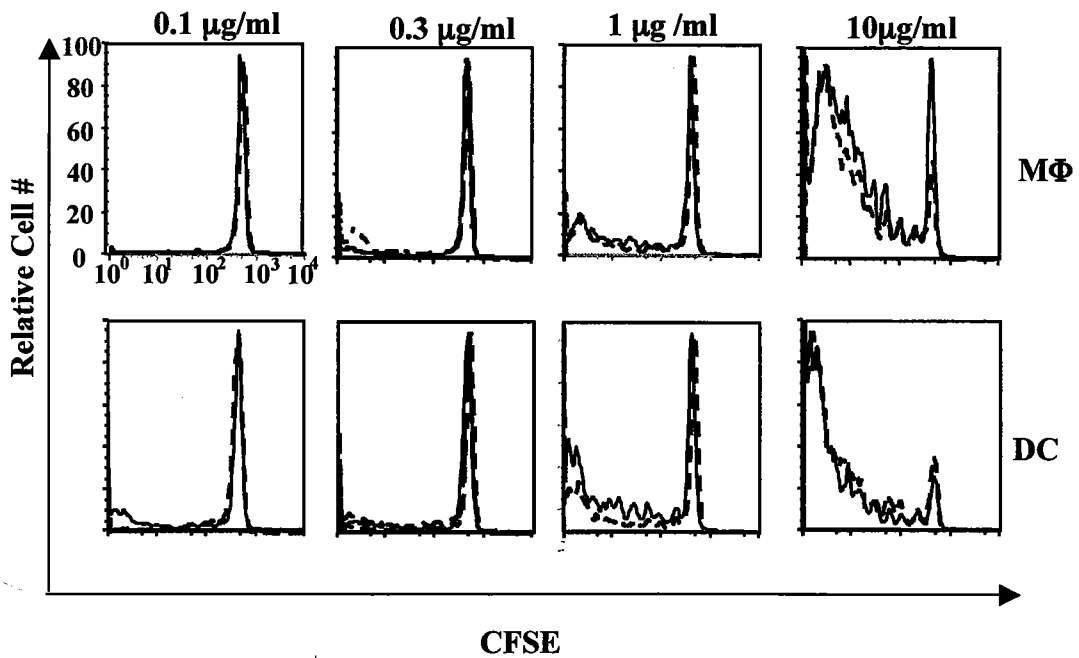


Figure 10B: Macrophages Prime Naïve T Cells As Well As DCs When Injected Intravenously



We had reasoned that intravenous injection of the APC would result in similar numbers of migrating cells, so we decided to quantitate the number of cells migrating into the secondary lymphoid organs after SC and IV immunization. We found that just about equal numbers of DCs and MΦs were found in the spleen after IV injection (Fig 11B). Quite a different picture was observed for the SC injections. After SC injection, we observed ~8-fold more DC than MΦ in the draining lymph nodes in this experiment (range for other experiments 5-10-fold). The results from a representative experiment can be seen in Fig. 11A. We were unable to reliably detect the migration of either APC to the inguinal lymph nodes when delivered intravenously.

Peptide-Pulsed T Cells Are Unable To Stimulate Responses

To determine if the capability of MΦs to prime naïve transgenic T cells is relatively unique or if any MHC class I positive cell can prime naïve transgenic T cells, we investigated whether T cells could also function as APCs in this model. T cells were chosen because they express class I molecules at high levels, can present peptide and migrate into the T cell areas of the secondary lymphoid organs. As a source of T cell APCs in these experiments, $CD8^+$ T cells were isolated from the lymph nodes of OT-I mice bred on to a Rag-deficient background. These mice were used to provide a homogeneous pure population of T cells of irrelevant specificity that lacked any possible B cell contamination. The OT-I T cells were pulsed with gp33 and injected *SC* into mice containing CFSE-labeled P-14 T cells. The pure population of $CD8^+$ T cells was completely unable to stimulate the naïve P-14 T cells to proliferate or expand (Fig. 12). In contrast, in the same experiment MΦs and DCs stimulated P-14 T

Figure 11: Dendritic Cells Migrate More Efficiently Out Of The Subcutaneous Space Than Macrophages.

A. $10x10^6$ CFSE labeled MΦs or DCs were injected SC. Twenty-four hours later, popliteal lymph nodes were removed and digested with collagenase and DNAse for 90 minutes. The nodes were made into single cell suspensions by pulling through a 25 G needle. Lymph nodes from an animal that did not receive any APC were used as negative controls. Samples were then analyzed by FACS analysis. The mean for the MΦs samples= 102.2 and the mean for the DC samples =798.7. These experiment is representative of 5 experiments with a total n=14 for MΦs and an n=11 for DCs.

B. **DCs And MΦs Migrate to the Spleen With Equal Efficiency When Injected IV.**

B. $10x10^6$ CFSE labeled MΦs or DCs were injected intravenously. Twenty-four hours later, spleens and inguinal lymph nodes were removed and digested with collagenase and DNAse for 90 minutes. The organs were made into single cell suspensions by pulling through a 25 G needle. A spleen from an animal that did not receive any APC was used as negative controls. Samples were then analyzed by FACS analysis. The mean for the MΦ samples= 2474.5 and the mean for the DC samples =2298.6. These experiment is representative of 5 experiments with a total n=15 for MΦs and an n=9 for DCs.

Figure 11A: Dendritic Cells Migrate To LNs After SC Injection More Efficiently Than Macrophages

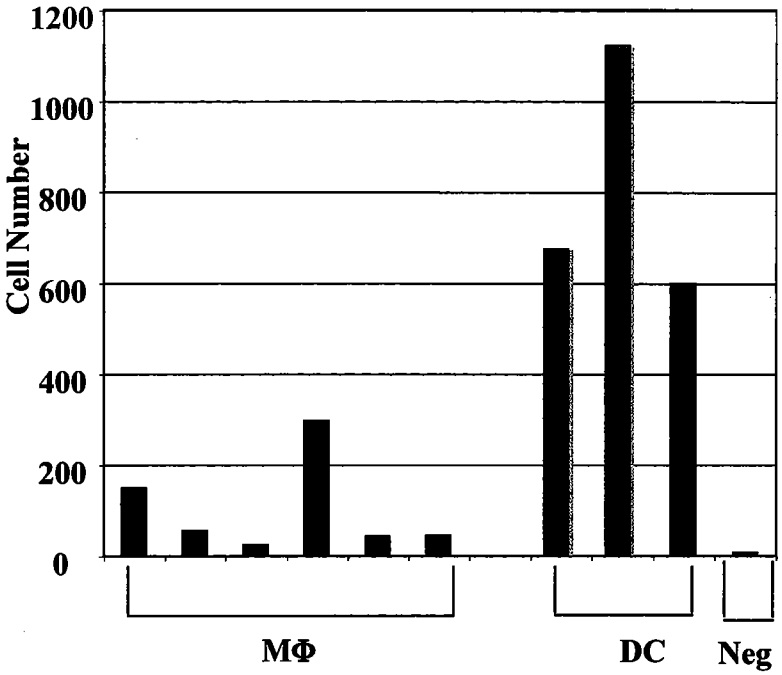


Figure 11B: Dendritic Cells And Macrophages Migrate With Equal Efficiency When Injected IV

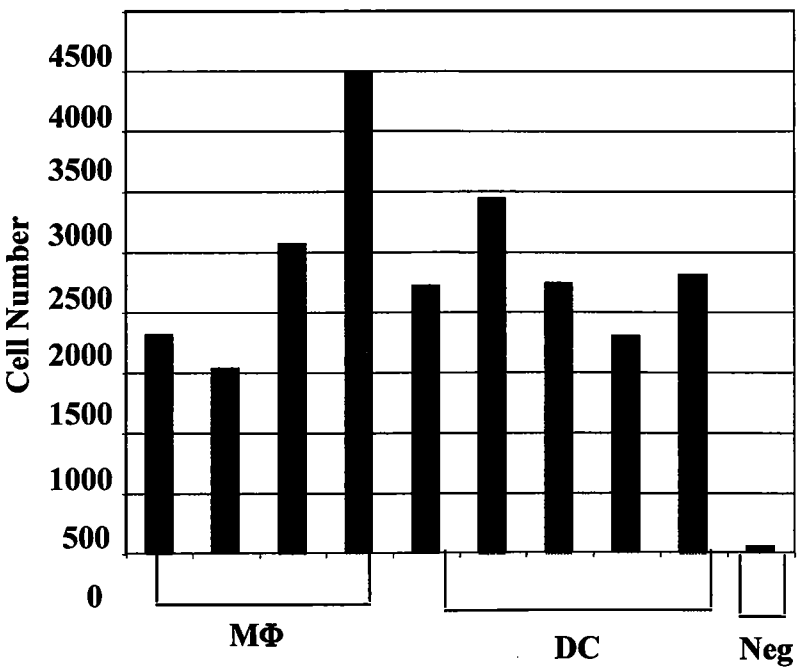
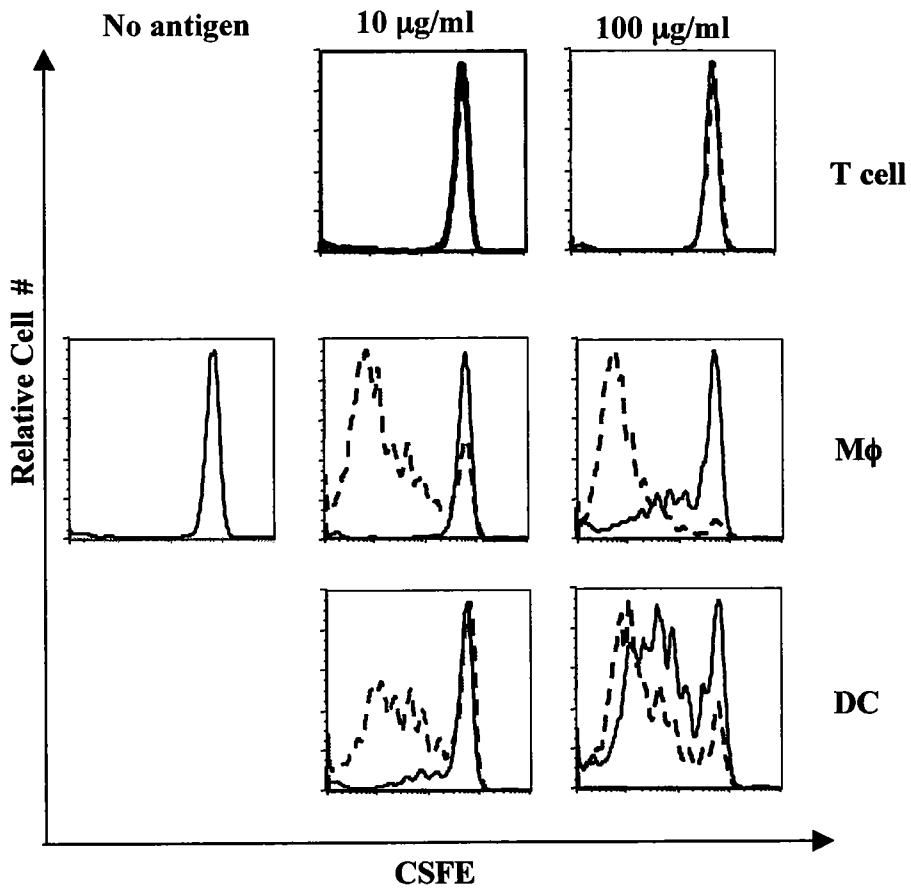


Figure 12: Subcutaneously Injected Peptide-pulsed T Cells Are Unable To Prime Naive P-14 $CD8^+$ T Cells.

$2.5x10^6$ CD45.1 congenic P-14 were CFSE labeled and adoptively transferred into C57BL/6 mice. One day later, the mice were immunized with $Rag^{-/-}$ OT-I T cells, MΦs or DCs pulsed with 0.0, 10 or 100 μg/ml of gp33. Three days later, draining lymph nodes were removed and stained with CD8 and CD45.1 and analyzed by FACS. The data are shown as CFSE content of the $CD8^+$ $CD45.1^+$ T cells. The different color lines represent different animals in the same group. This experiment was repeated 3 times with a total n=8 mice/group.

Figure 12: T Cells injected SC Are Unable to Prime Naïve P-14 CD8 $^{+}$ T Cells



cells demonstrating that the CFSE labeled P-14 T cells were capable of proliferating (Fig. 12). As a control for migration, T cells from GFP transgenic mice were injected subcutaneously into the footpad of mice. By cryosections and fluorescent microscopy, we have found that the T cells do migrate to the draining lymph node (data not shown). To further verify that the failure of gp33 pulsed Rag-/OT-I T cells to induce proliferation of P-14 TCR transgenic T cells was not due to their inability to home to the draining lymph node in sufficient numbers, we injected the OT-I T cell APCs intravenously. Under these conditions the T cell "APCs" migrate efficiently to the secondary lymphoid organs, however as shown in Fig. 13, they are again very poor APCs. Although the peptide-pulsed T cells were able to induce a small amount of proliferation of the P-14 T cells, no more than 3 divisions were observed as seen in Fig. 13. There were also no P-14 T cells accumulating in the later division peaks. Evidence will be presented in the next chapter that this very limited response is not being directly stimulated by the OT-I T cells, but by cross-presentation. Therefore, not all MHC class I expressing cells can stimulate naïve T cells, even when the "APCs" can home to the T cell areas in the lymphoid organs.

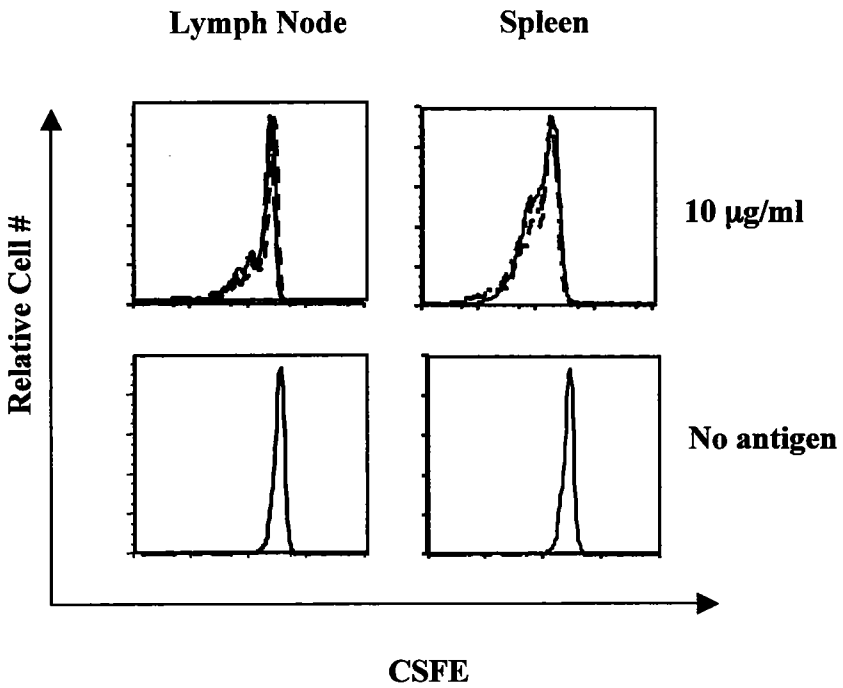
Macrophages And Dendritic Cells Stimulate The Generation Of Effector T Cells.

Effective immune responses require that T cells not only proliferate but also express effector functions. Under some conditions, T cells can be stimulated to undergo

Figure 13: Peptide-Pulsed T Cells Injected Intravenously Are Very Poor APCs.

$2.5x10^6$ CD45.1 congenic P-14 were CFSE labeled and adoptively transferred into C57BL/6 mice. One day later, the mice were immunized with OT-I/$Rag^{-/-}$ T cells, MΦs or DCs pulsed with 0.0 or 10μg/ml of gp33. Three days later, spleens and inguinal lymph nodes were removed and stained with CD8 and CD45.1 and analyzed by FACS. The different color lines represent different animals in the same group. This experiment is representative of 3 experiments with a total n=6 mice/group.

Figure 13:Peptide-Pulsed T Cells Injected Intravenously Are Very Poor APCs.



many rounds of cell division but become anergic and fail to express effector functions (Adler et al., 2000). In order to determine if both DCs and MΦs stimulate naïve P-14 T cells to become effector cells, we first analyzed whether the proliferating T cells were producing the effector cytokine IFN-γ. Adoptively transferred P-14 T cells were stimulated *in vivo* with peptide-pulsed MΦs or DCs administered SC for the OT-I experiment and IV for the P-14 experiment as described above. Three or four days after the APC transfer, the draining lymph nodes were removed made into single cell suspensions and cultured *in vitro* with or without gp33 or OVA-p peptide in the presence of Brefeldin A for 5 hours prior to staining for intracellular IFN-γ. Shown in Table 1 and Fig.14, both MΦs and DCs stimulated naïve P-14 T and OT-I cells to produce IFN-γ. Although fewer T cells are primed by the SC administered MΦs (Fig. 14), the primed T cells are making the effector cytokine and are therefore functional. When the APC were given IV (Table 1), the percentage of T cells making IFN-γ was similar for both APCs. Consistent with previous reports, only T cells that have under gone multiple rounds of division are making significant amounts of IFN-γ.

We next analyzed the ability of both MΦs and DCs to induce cytolytic function using *in vivo* cytolysis assays as described (Barber et al., 2003). In these experiments, unlabeled P-14 T cells were adoptively transferred into C57BL/6 mice. The next day, gp33-pulsed MΦs or DCs were given IV. Various days after APC transfer, CD45.1 congenic gp33-pulsed and unpulsed splenocytes labeled with two different CFSE concentrations were given IV.

Table 1: P-14 T Cells Produce IFN-γ After Priming *In Vivo* In C57BL/6 Mice.

CSFE labeled CD45.1$^+$ P-14 T cells were adoptively transferred into C57BL/6 mice. One day later, the mice were immunized IV with MΦs or DCs pulsed with 10 μg/ml. Three days after immunization, splenocytes were incubated with 5 μg/ml of gp33 or media as a control for 5 hours in the presence of Brefeldin A. Samples were then stained for CD45.1, CD8 and intracellular IFN-γ. The data were gated on CD8$^+$, CD45.1$^+$ cells and expressed as % transgenic T cells producing the cytokine. This experiment is representative of 3 experiments with a total n=6 mice/group.

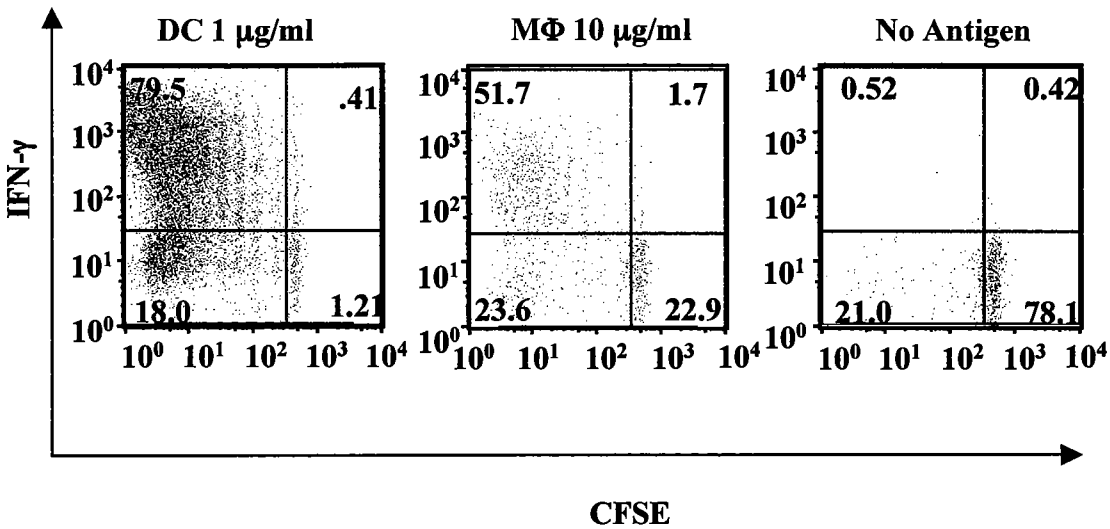
Table 1: P-14 T Cells Produce IFN-γ After Priming *in Vivo* in C57/BL6 Mice

Stimulating APC	% Transgenic T cells secreting IFN-γ
No Antigen	1.54
DC 10 μg/ml peptide mouse 1 DC 10 μg/ml peptide mouse 2	37.1 31.5
MΦ10 μg/ml peptide mouse 1 MΦ10 μg/ml peptide mouse 2	30.1 27.3

Figure 14: IFN-γ Secretion Of OT-I T Cells Primed *In Vivo.*

CSFE labeled CD90.1$^+$, P-14 T cells were adoptively transferred into C57BL/6 mice. One day later, the mice were immunized SC with MΦs pulsed with 10 μg/ml or DCs pulsed with 1.0 μg/ml of OVA-p. Four days after immunization, draining lymph node cells were incubated with 1.0 μg/ml OVA-p or media as a control for 5 hours in the presence of Brefeldin A. Samples were then stained for CD90.1, CD8 and intracellular IFN-γ. The data were gated on CD8, CD90.1$^+$ cells and plotted as transgenic T cells producing IFN-γ versus CFSE content. This experiment is representative of 3 experiments with a total n=6 mice/group.

Figure 14: IFN-γ Secretion by OT-I T cells on Day 4



Eighteen hours later, the animals were sacrificed and spleens were removed and analyzed by FACS. Both DCs and MΦs were able to prime CTL effector cells that lysed the peptide-pulsed targets. Cytolytic activity was seen as early as 2 days after priming and reaches a maximum by day 4. We observed no cytolytic activity after day 12 in primed animals (data not shown). On day 5, two animals primed with DCs pulsed with 10 μg/ml of gp33 peptide displayed 87 and 85 % specific lysis of peptide-pulsed targets respectively (Table 2). MΦs coated with 10 μg/ml of peptide were able to induce 48 and 60% specific lysis respectively in two animals (Table 2). In this experiment the background level of killing in animals primed with non-pulsed APCs was 4% in one animal and 0.3 % in the other animal. Taken together the intracellular IFN-γ and the *in vivo* cytolysis assays indicate that MΦs and DCs are both able to stimulate naïve P-14 T cells to become fully functional effector cells.

Macrophages And Dendritic Cells Can Stimulate The Generation Of T Cell Memory

Even though priming with MΦs leads to the formation of a pool of effector cells, it was still a formal possibility that the stimulation of naïve T cells by MΦs would not lead to the formation of memory T cells. To evaluate this issue, we analyzed mice 40 days after they were injected with CFSE-labeled transgenic P-14 T cells and primed with peptide-pulsed or unpulsed APCs.

Table 2: P-14 T Cells Become Cytolytic After *In Vivo* Priming.

P-14 T cells were adoptively transferred into C57BL/6 mice. One day later, the animals were immunized with MΦs or DCs pulsed with 10 μg/ml of gp33. Four days after immunization, spleens from congenic mice were harvested and divided into 2 populations: one population was CSFE labeled and unpulsed and the other population was labeled with one-third the amount of CFSE and pulsed with gp33. $10x10^6$ pulsed and $10x10^6$ unpulsed splenocytes were mixed together and injected IV into the primed hosts. After 18 hours, the spleens were removed and stained for CD45.1 and analyzed by FACS. Percent lysis was calculated as described in the Materials and Methods.
This experiment is representative of 3 experiments with a total n=9 mice/group.

Table 2: Induction of Cytolytic Activity in P-14 T Cells After *In Vivo* Priming

Sample	% Lysis
Control	4% 0.3%
DC 10 µg/ml	87% 85%
Mø 10 µg/ml	48% 60%

Since intravenous inoculation of APCs led to a larger pool of effectors, we chose this route of immunization for these experiments. Memory cells were identified as transferred T cells that had undergone a complete loss of CFSE and survived >40 days (Asano and Ahmed, 1996). Table 3 shows the results from two mice. In animals injected with gp33-pulsed MΦs 10.5 and 24.8 % of the P-14 T cells in the lymph nodes had gone on to become memory cells. The percentages of P-14 memory cells in the spleen were slightly lower in these same animals, 2.2 and 15.1%, respectively. The percentages of memory transgenic T cells observed in the animals primed with intravenously administered DCs were 5.9 and 8 % in the spleen. Slightly higher frequencies of memory cells were observed in the lymph nodes of these same animals, 8.6 % and 13.7 % respectively. Very few CFSE negative P-14 T cells were identified in the mice injected with unpulsed DCs or MΦs, as expected. These data demonstrate that MΦs and DCs prime similar numbers of memory cells.

To test whether MΦs primed memory cells were functional, we stimulated these cells *ex vivo* with 5μg/ml peptide for 5 hours and performed intracellular IFN-γ cytokine staining. Results from a typical experiment are shown in Table 4. In the animals receiving unpulsed APCs less than 1.5 % of the transgenic T cells could be stimulated to synthesize IFN-γ in this time period in either the lymph nodes or the spleen. In contrast, animals that received gp33-pulsed MΦs or DCs had a substantial population (between 18-35 %) of IFN-γ secreting transgenic P-14 T cells. Therefore, MΦs and DCs stimulate the generation of similar levels of functional memory cells.

Table 3: Priming Of P-14 T Cells By Macrophages Leads To Memory Cell Formation.

CFSE-labeled P-14 T cells congenic for CD90.1 were adoptively transferred IV into C57BL/6 mice. On day later, $1x10^6$ APCs were given IV. After forty days, spleens and inguinal lymph nodes were removed, stained and analyzed by FACS. Memory cells were defined as $CD8^+CD90.1^+ CSFE^-$ present in the lymphoid organs after 40 days. The data from a representative experiment are presented as % of CD8 cells that are memory cells. This experiment was repeated 3 times with a total n=10 mice/group.

Table 3 : Priming of P-14 T Cells With MΦ Leads to the Formation of Memory Cells

Immunizing APC	% memory cells in lymph node	% memory cells in spleen
DC 10μg/ml mouse 1	8.6	5.86
DC 10μg/ml mouse 2	13.7	7.97
MΦ 10μg/ml mouse 1	24.8	15.1
MΦ 10μg/ml mouse 2	10.5	2.23
No Antigen	1.68	1.69

Table 4: Priming of P-14 T Cells by Macrophages Leads to Functional Memory.

CFSE labeled CD90.1$^+$ P-14 T cells were adoptively transferred into C57BL/6 mice. One day later, MΦs or DCs pulsed with 10 μg/ml of gp33 were given intravenously. Forty days later, spleens and lymph nodes were removed and stained for CD8. CD90.1 and intracellular IFN-γ. The data are represented as % of CD8$^+$ CD90.1$^+$ CFSE$^-$ cells secreting the cytokine. This experiment was repeated 3 times with a total n=6 mice/group.

Table 4: Priming of P-14 T Cells by MΦ Leads to Functional Memory

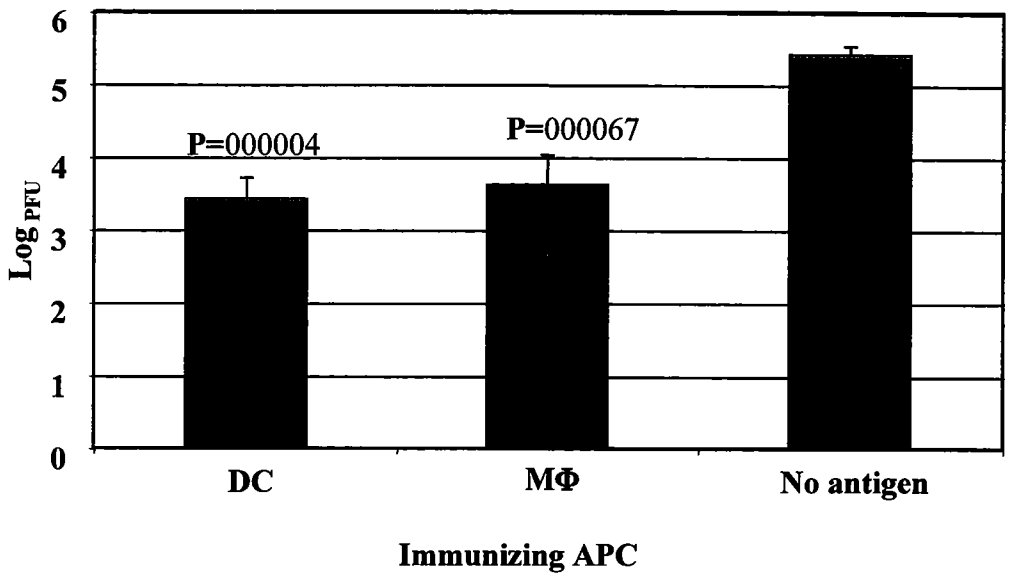
APC Type	% IFN^+P-14 T cells in Lymph Node	% IFN^+P-14 T cells in Spleen
No Antigen	1.02	1.03
DC 10 μg/ml	18.4	35.1
MΦ 10 μg/ml	24.2	32.6

To determine whether or not MΦs could prime endogenous polyclonal nontransgenic T cells, C57BL/6 mice were immunized intravenously with either MΦs or DCs pulsed with 10 μg/ml of the gp33 peptide. Mice were rested for 40 days to allow for the formation of memory T cells. The mice were then challenged with $5x10^4$ plaque forming units of LCMV. Four days later spleens were harvested and homogenized and plaque assays were performed. The results from a typical experiment can be seen in Fig.15. Immunization with either MΦs or DCs led to partial protection in these mice. These data demonstrate that MΦs can prime naive T cells and that these primed T cells can offer protection to a viral challenge.

Figure 15: Both Macrophages and Dendritic Cells Induce Protective Immunity.

C57BL/6 mice were immunized with either MΦs or DCs pulsed with 5μg/ml of gp33 peptide. Mice were rested for 40 days to allow for the formation of memory T cells. The mice were than challenged with $5x10^4$ plaque forming units of LCMV. Four days later spleens were harvested and homogenized and plaque assays were performed. The data are represented as $Log_{PFU.}$ Student T tests were performed and the data were statistically significant p= .000004 for DCs and p= .000067 for MΦs when compared to the animals immunized with unpulsed APCs. The difference in protection offered between the 2 APC types was not statistically significant p=0.56. This experiment is representative of 3 experiments with a total n=13 mice/group.

Figure 15 :Both APCs Induce Protective Immunity



Chapter IV: Macrophages And Dendritic Cells Directly Prime Naïve $CD8^+$ T Cells

Results

Visualization Of APC-T Cell Clusters

Since in wild type mice the possibility of priming of the transgenic T cells by host APC exists, we decided to perform a series of experiments to examine the role of cross-presentation. The first indication we had that the transferred APCs were actually the ones priming the transgenic T cells came from fluorescent microscopy studies. Using T cells labeled with Cell Tracker green and APCs labeled with Cell Tracker orange, we were able to observe the clustering of the transgenic T cells with the adoptively transferred DCs and MΦs. Figure 16 panel A shows a representative cluster of a transferred SIINFEKL-pulsed DC with OT-I T cells and panel B shows a SIINFEKL-MΦ OT-I T cell cluster. This figure also allows for the visualization of some T cell division indicated by the dimmer levels of green fluorescence in some T cells.

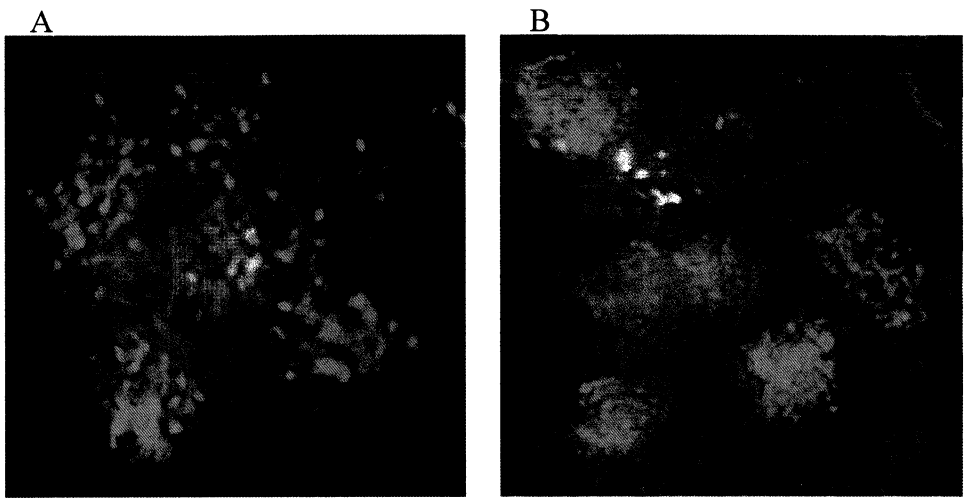
Macrophages And Dendritic Cells Directly Prime $CD8^+$ T Cells In F1 Chimeric Mice

While the studies above suggest that the transferred APCs are responsible for the antigen presentation, it was still possible that cross-presentation, the transferring of peptide to the host APCs occurred. To distinguish between the possibilities, we used mice whose bone marrow- derived APCs lack the appropriate MHC I molecule needed to present the peptide potentially transferred from the injected MΦs. In one set of experiments we used radiation bone marrow chimeras. H-2^b x H-2^s (bxs) F1 mice were lethally irradiated and reconstituted with bone marrow from mice that were either H-2^b or H-2^s.

Figure 16: Visualization of Antigen Presenting Cell and T cell Interactions.

$2.5x10^6$ CFSE labeled $CD90.1^+$ OT-I T cells were adoptively transferred into C57BL/6 mice. One day later, the mice were immunized SC with MΦs or DCs pulsed with OVA-p. Twenty-four hours (panel A) and forty-eight hours (panel B the draining lymph nodes were removed and frozen in a dry ice ethanol bath. 25 μm cryosections were made and examined by fluorescent microscopy and analyzed by Z-axis deconvolution software.

Figure 16 : Visualization of Antigen Presenting Cell and T Cell Interactions



OT-1 T cell and DC cluster **OT-1 T cell and MΦ cluster**

=APC **=T CELL**

APCs from H-2^s mice are unable to present KAVYNFATC whereas H-2^b APCs can present this peptide (Fig. 17). The chimeric mice were allowed to rest for 3-4 months and the replacement of their APCs was tested by adoptively transferring CFSE -labeled TCR transgenic T cells and immunizing with the 13mer (the LCMV peptide KAVYNFATCGIFA). This peptide has previously been shown to be able to prime T cells and can be cross-presented on H-2^b (Ciupitu et al., 1998). P-14 transgenic T cells were stimulated to proliferate in F1 mice that were reconstituted with H-2^b bone marrow and immunized with exogenous antigens (Fig. 18). In contrast, the transgenic T cells failed to be stimulated by these antigens in chimeras reconstituted with H-2^s bone marrow (Fig. 18). These results demonstrated that the F1 host's bone marrow-derived APCs had been completely replaced and consequently the s→bxs chimeras were unable to cross-present antigen on H-2^b class I molecules.

We next tested whether transferred P-14 T cells were stimulated in chimeric mice by KAVYNFATC-pulsed MΦs or DCs that were injected intravenously. Figure 19 demonstrates that MΦs are able to stimulate proliferation of CFSE-labeled P-14 T cells in the H-2^s BM reconstituted chimeras. In this figure the different traces represent individual mice. The variability between mice seen in this experiment is typical and maybe due to the variability in injection of the bone marrow used to reconstitute these mice. In some experiments the ability of both DCs and MΦs to stimulate P-14 T cells to proliferate was slightly reduced in the s→ bxs chimeras as compared to C57BL/6 mice, but not different from that seen in the b→bxs chimeras; presumably this was attributable to an effect of radiation and/or bone-marrow reconstitution.

Figure 17: Generation of F1 Chimeras.

This is a schematic representation of how the F1 chimeras were generated. B6XSJL F1 mice were lethally irradiated. Twenty-four hours later the mice were reconstituted with T cell-depleted bone marrow from either C57BL/6 or B10.S-$H2^s$ mice. The mice were housed for 3.0-4.0 months to allow for reconstitution of all bone marrow derived APCs.

Figure 17 : Construction of $H2^s$ → B6SJL F1 Bone Marrow Chimeras

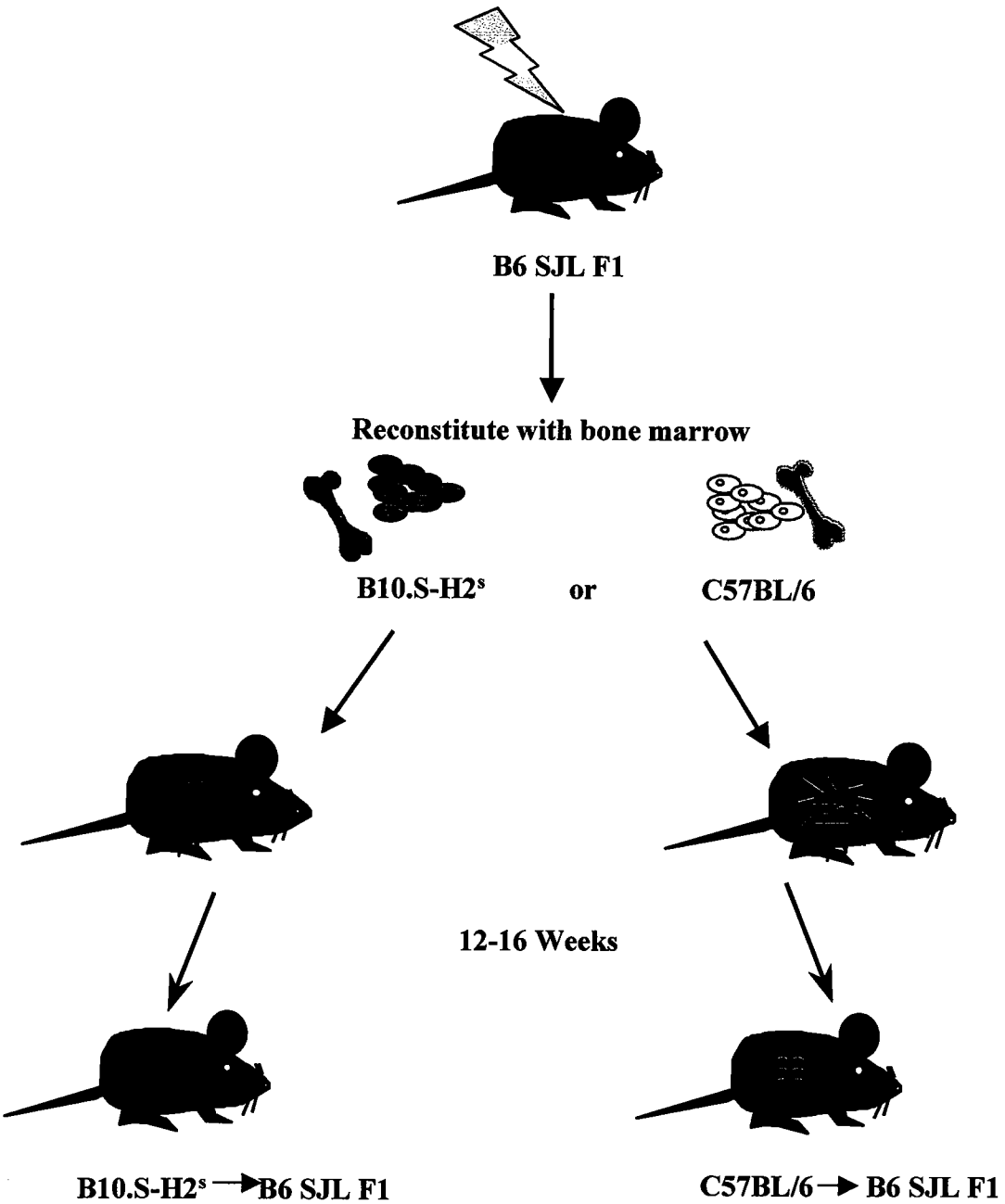


Figure 18: Test of Chimera Reconstitution.

$3.0x10^6$ CFSE-labeled CD90.1$^+$ P-14 T cells were transferred into bone marrow chimeras. One day later, the mice were immunized with 1.0 $x10^6$ APCs IV. Four days later, inguinal lymph nodes were removed and stained with APC conjugated anti-CD90.1 and PerCP conjugated anti-CD8. The above panels are gated on CD8$^+$, CD90.1 $^+$ live cells. The different colored lines represent different animals in the same experiment. In every experiment 2 animals were immunized with the 13 mer to test for chimera reconstitution.

FIGURE 18: Test of LCMV Chimeras

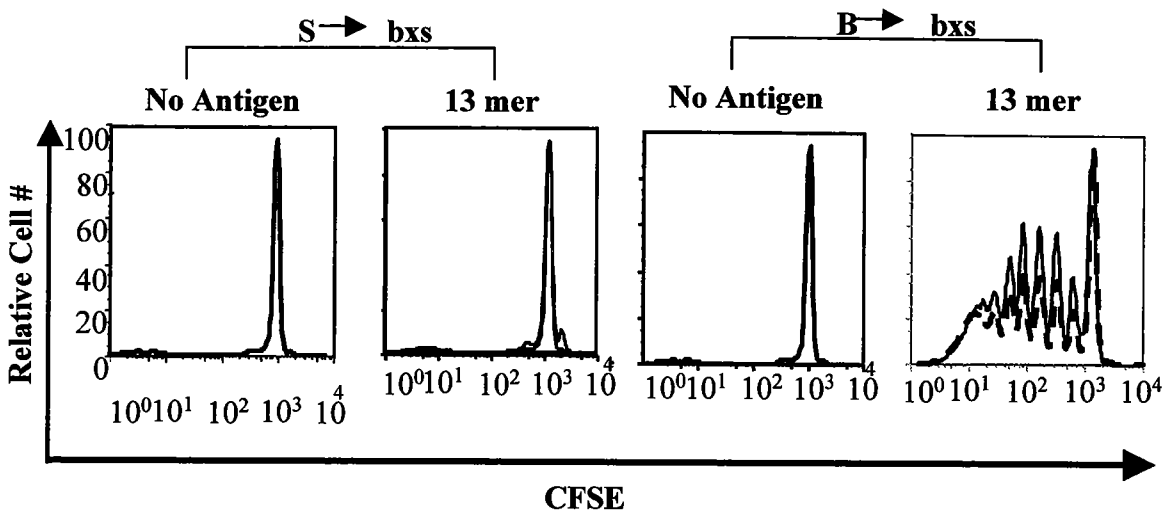
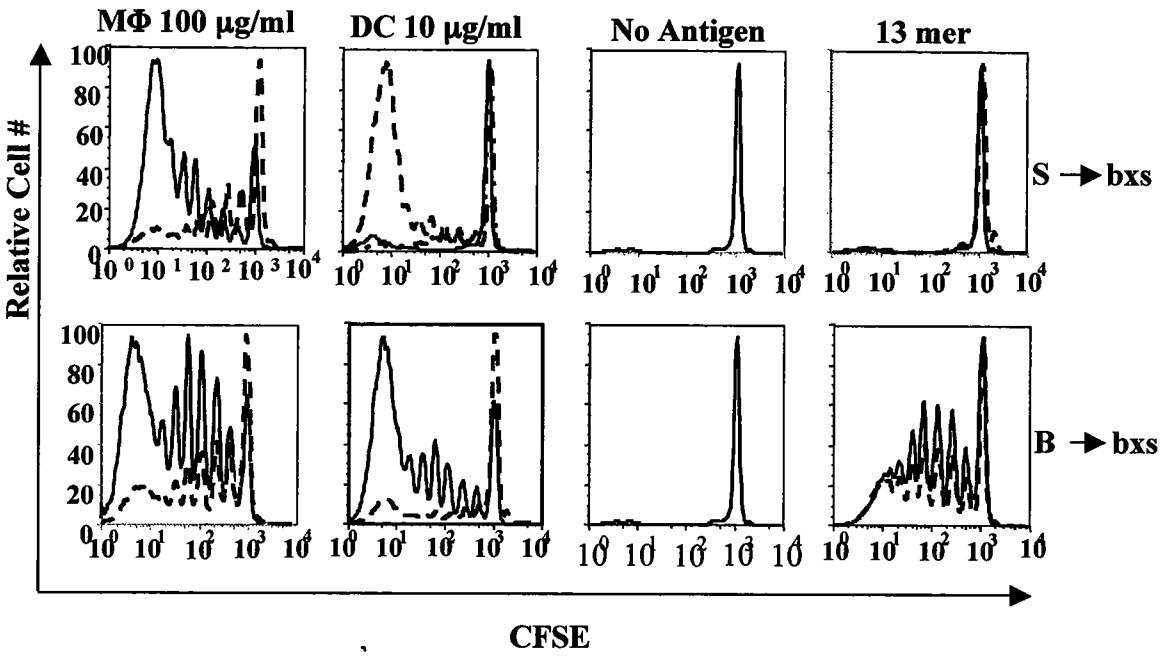


Figure 19: Macrophages Prime Naïve P-14 T Cells in Bone Marrow Chimeras.

$3.8x10^6$ CFSE-labeled CD90.1$^+$ P-14 T cells were transferred into bone marrow chimeras. One day later, the mice were immunized with $0.5x10^6$ APCs in the footpad. Unpulsed APCs and the LCMV 13mer were used as the negative and positive control. Four days later, the draining lymph nodes were removed and stained with APC conjugated anti-CD90.1 and PerCP conjugated anti CD8. The above panels are gated on CD8$^+$, CD90.1 $^+$ live cells. The different colored lines represent different animals in the same experiment. This experiment was repeated 6 times with a total n=24 mice/group.

Figure 19: Macrophages Prime Naïve P-14 T Cells in Bone Marrow Chimeras



Since the s→bxs chimeras lack bone marrow-derived APCs that can present peptide on D^b, we conclude that the peptide-pulsed MΦs are directly priming the T cells in these bone-marrow chimeras.

To establish that the P-14 transgenic T cells directly primed in the chimeric mice differentiated into effector T cells, we analyzed whether they were producing IFN-γ by intracellular staining. One day after P-14 T cell transfer, chimeric mice were immunized intravenously with gp33 pulsed APCs. Three days later, the mice were sacrificed and intracellular cytokine staining was done on the *in vitro* restimulated splenocytes. Priming with both MΦs and DCs stimulated P-14 transgenic T cells to differentiate into IFN-γ secreting effector T cells in s→bxs chimeras (Fig. 20). In agreement with the results seen in wild type mice, only P-14 cells that had undergone multiple rounds of cellular division were capable of producing significant amounts of this cytokine in this assay (Fig. 20). If these primed chimeric animals were rested for 40 days, P-14 functional memory cells were also present and able to make IFN-γ (Table 5).

Macrophages And Dendritic Cells Directly Prime T Cells In β2m "Knockout" Mice

To further examine the impact of cross-priming in these responses, we transferred CFSE-labeled P-14 T cells into β2-microglobulin deficient mice, whose bone-marrow-derived APCs and other cells lack MHC I molecules. The transgenic P-14 T cells did not proliferated in the β2-microglobulin knockout mice when they were immunized with the 13mer but did proliferate in C57BL/6 mice injected with this peptide (Fig. 21).

Figure 20: Macrophages Directly Prime P-14 T Cells To Produce IFN-γ.

CFSE-labeled CD90.1^{+} P-14 T cells were transferred into bone marrow chimeras. One day later, the mice were immunized with 0.5x10^{6} APCs in the footpad. Unpulsed APCs and the LCMV 13mer were used as the negative and positive control. Four days later, the draining lymph nodes were removed and stained with APC-conjugated anti-CD90.1 and PerCP conjugated anti CD8. The above panels are gated on CD8^{+}, CD90.1 $^{+}$ live cells. The data are plotted as CFSE content of transgenic cells producing IFN-γ. The numbers in the left hand corner are percentage of transgenic cells producing IFN-γ. This experiment is representative of 3 experiments with a total n=5 mice/group.

Figure 20: Macrophages and Dendritic Cells Directly Prime P-14 Effector Function

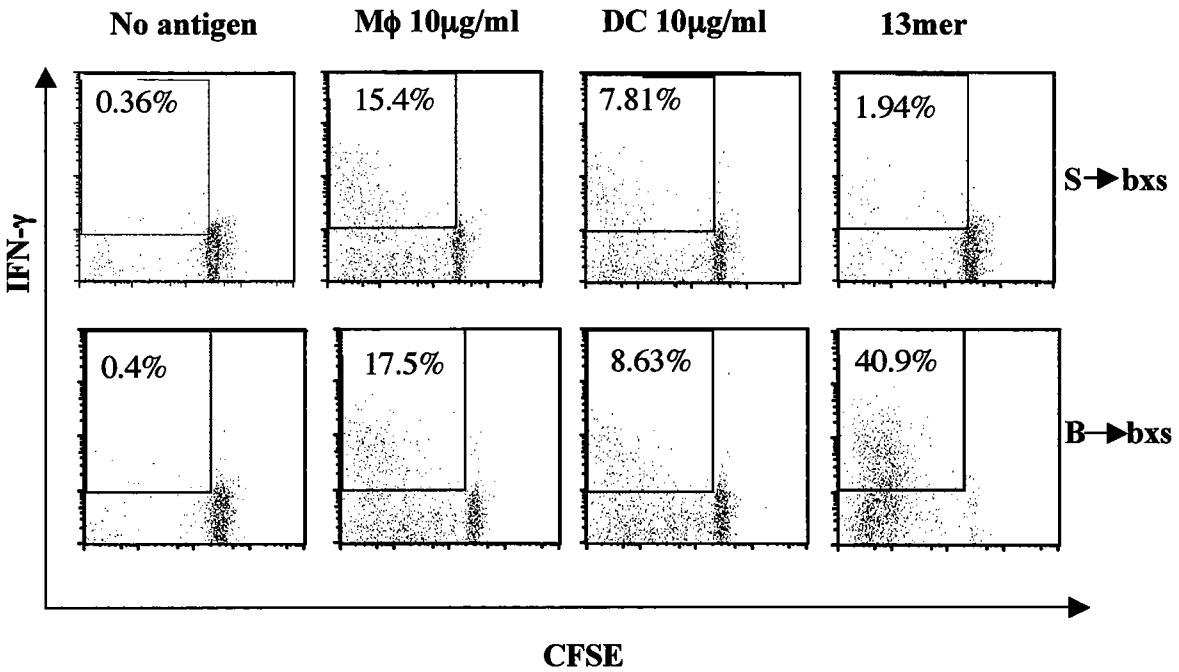


Table 5: P-14 $CD8^+$ T Cells Primed By Macrophages Differentiate Into Functional Memory Cells In Bone Marrow Chimeras.

$CD90.1^+$ CSFE–labeled P-14 T cells were adoptively transferred into BM chimeras. One day later, the mice were immunized IV with $1x10^6$ MΦσ or DC pulsed with 10μg/ml of gp33 peptide or 5 μg/ml of the 13mer as a positive control. Forty days later, inguinal lymph nodes and spleens were removed and stimulated *in vitro* with 5μg/ml gp33 in the presence of Brefeldin A. The samples were then stained for CD90.1, CD8 and intracellular IFN-γ and analyzed by FACS. These data are shown as % P-14 T cells containing IFN-γ. This experiment is representative of 3 experiments with a total n=7 mice/group.

Table 5: Functional Memory Cells are Present in F1 Chimeras 40 days after Priming

	% IFN–γ + P-14 T cells in the Lymph Node	% IFN-γ + P-14 T cells in the Spleen
B6⟶ bxs 13 mer 5 μg	59.5	63.2
B6⟶ bxs DC Nag	1.49	1.75
B6⟶bxs DC 10 μg /ml	13.6	35.7
B6⟶ bxs MΦ 10 μg /ml	22.4	36.2
WT DC Nag	1.02	1.13
WT DC 10 μg /ml	18.4	35.1
WT MΦ 10 μg /ml	24.2	32.6
S⟶bxs 13 mer 5 μg	2.19	1.12
S⟶bxs DC 10 μg /ml	10.3	40.8
S⟶ bxs MΦ 10 μg/ml	18.6	34.5

These results confirm that the β2-microglobulin deficient mice are unable to cross-present antigen, as expected. Gp33-pulsed APCs were injected subcutaneously and three days later the draining lymph node were removed and analyzed by FACS. In this system, MΦs were able to stimulate the P-14 T cells at peptide concentrations of 10 and 100 μg/ml in β2m knockout mice, the same as observed in wild type mice (Fig. 21). The DCs were also able to prime naïve T cells in the β2-microglobulin deficient mice to the same level as in the wild type mice. In addition, in the β2-microglobulin deficient experiments DCs and MΦs stimulated P-14 T cell proliferation to the same extent. These experiments were also done using a peptide-pulsed macrophage and dendritic cell line. The results of this experiment can be seen in Fig. 22. As previously demonstrated in wild type mice, both cell lines were able to stimulate T cell proliferation, further supporting the ability of MΦs to directly prime naive T cells. To demonstrate that the P-14 T cells directly stimulated by MΦs became effectors, intracellular IFN-γ staining was performed as described above on β2m knockout mice that had been immunized with DCs and MΦs. Priming with both MΦs and DCs led to the differentiation of P-14 T cells into IFN-γ-secreting effectors (Figure 23). The percentage of terminally divided P-14 T cells able of making IFN-γ is similar irrespective of the APC doing the stimulating. Together with the chimera data, these results demonstrate that MΦs can directly stimulate naïve T cell responses.

Figure 21: Macrophages Directly Prime Naïve P-14 CD8+ T Cells In β2m Deficient Mice.

CD45.1 congenic P-14 T cells were CFSE-labeled and adoptively transferred into β2m deficient hosts. One day later, mice were immunized SC with DCs or MΦσ pulsed with 10 μg/ml or 100 μg/ml of gp33. The draining lymph node was removed 4 days later and stained for CD8 and CD45.1. The data are displayed as CFSE content of the live, $CD45.1^+$ and $CD8^+$ and the different colors represent the results of 2 or 3 different mice. This experiment was repeated 4 times with a total n=12 mice/group.

Figure 21: MΦs Directly Priming Naïve P-14 CD8$^+$ T Cells in β2m Deficient Mice

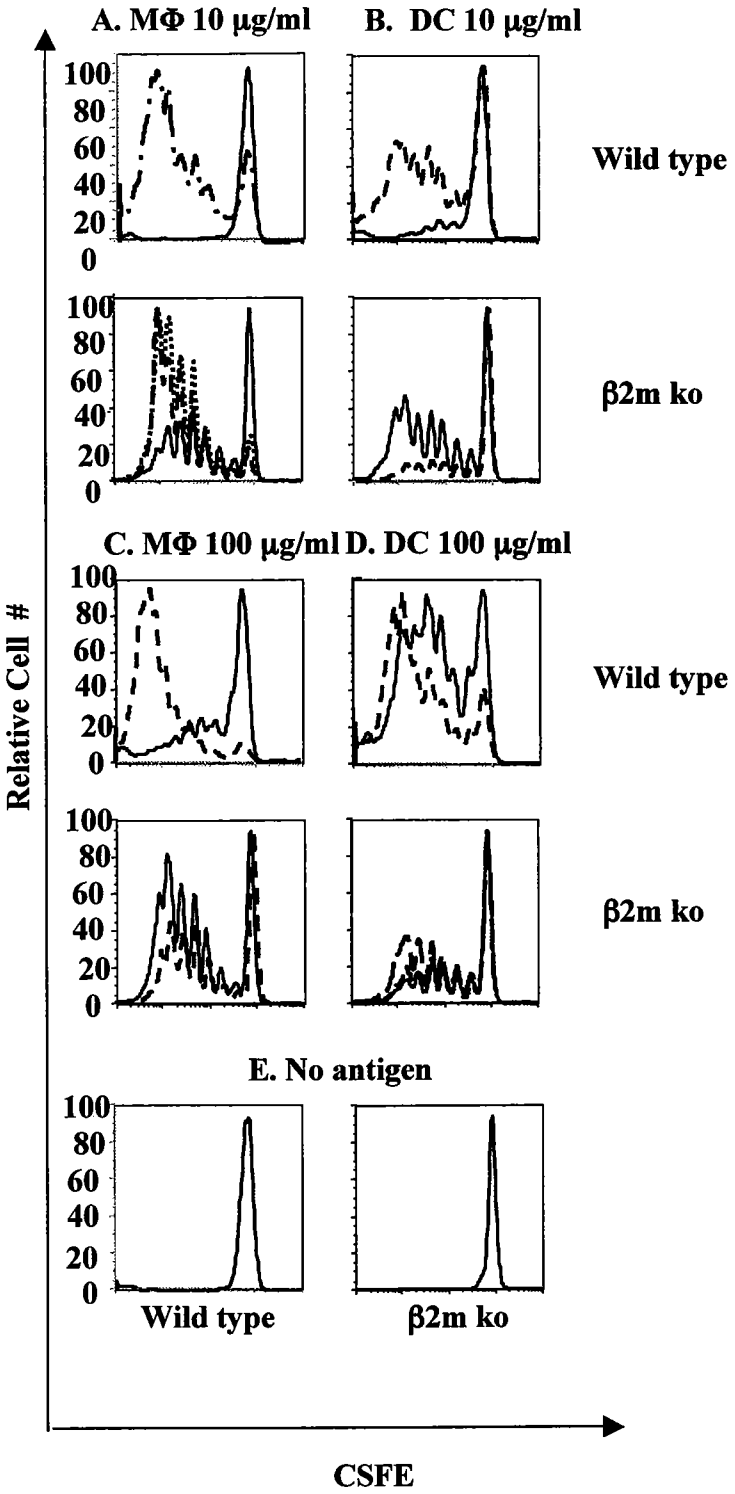


Figure 22: The MΦ And DC Cell Lines Directly Prime Naïve P-14 T Cells In β2m Deficient Mice.

$CD90.1^+$ T cells were CFSE-labeled and transferred into β2m knockout mice. One day later, C2.3 (a MΦ cell line) or DC2.4 (a DC cell line) cells pulsed with gp33 were injected SC. Four days later, popliteal lymph nodes were removed. The lymph node cells were analyzed by FACS. The data are represented as CFSE content of $CD90.1^+$, $CD8^+$ T cells. The different color lines represent different mice in the same experiment. This experiment was repeated 2 times with a total n=8 mice/group.

Figure 22: Cell Lines Directly Primes Naïve P-14 T Cells in β2m Deficient Mice

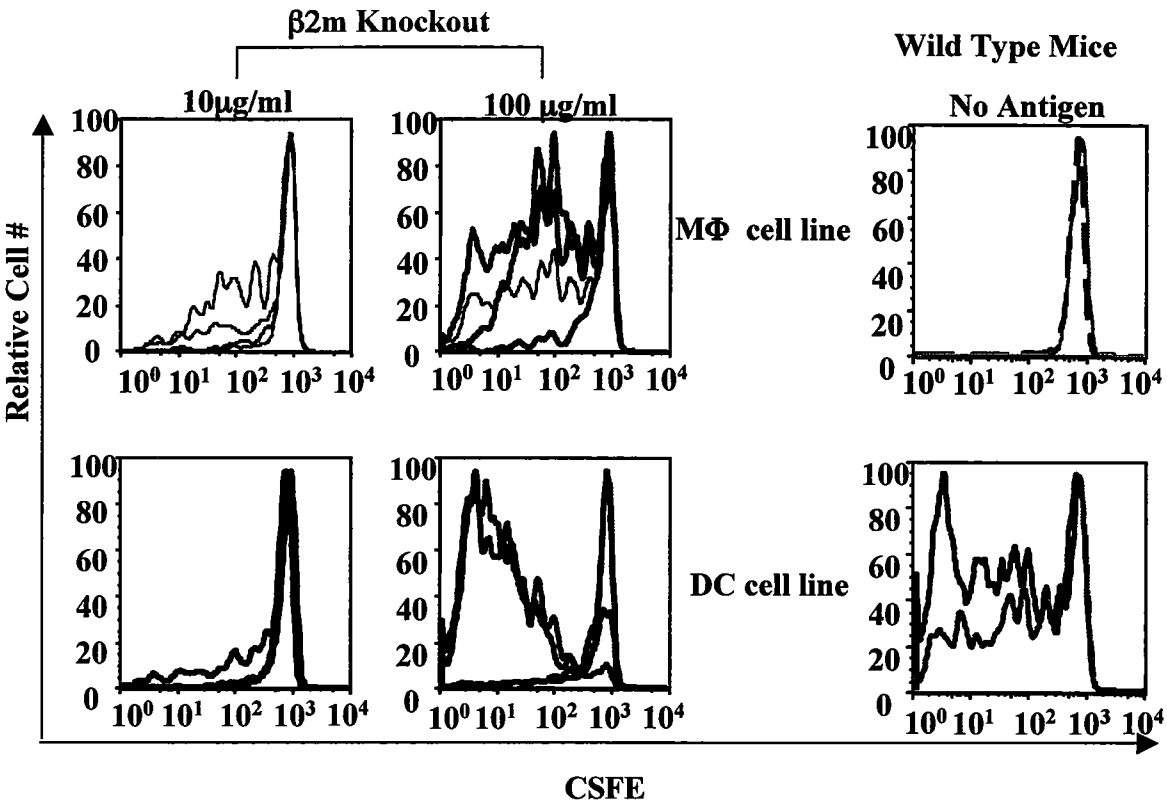
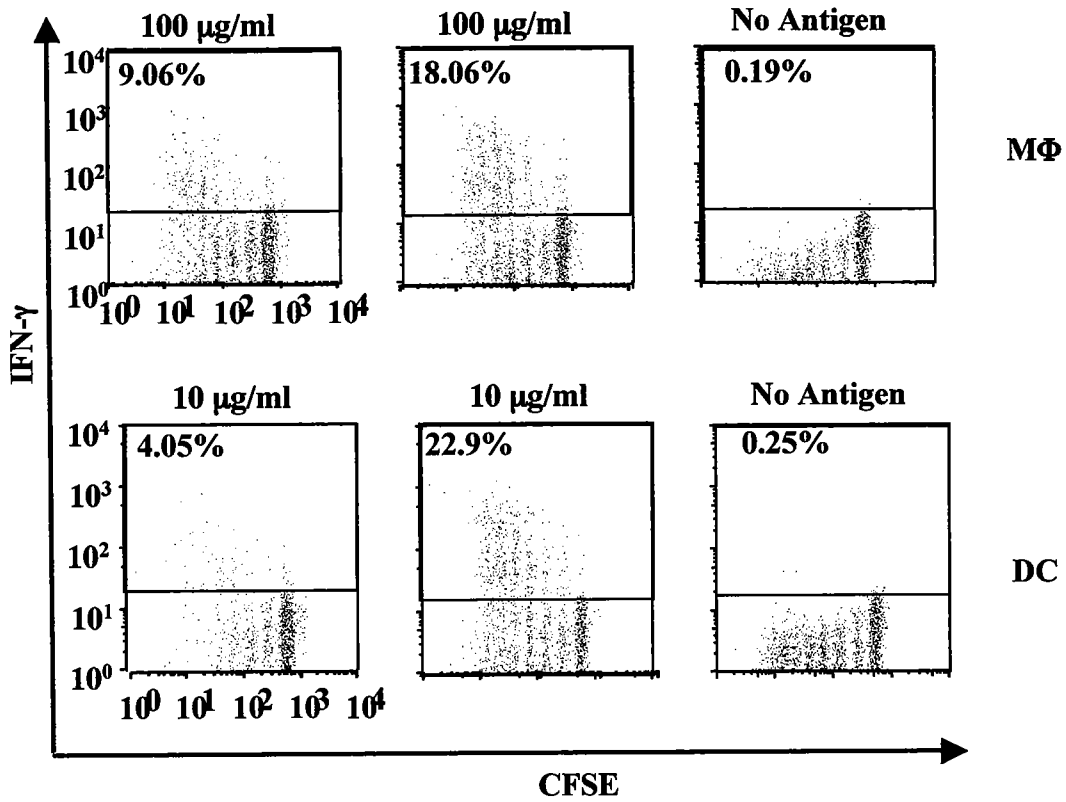


Figure 23: Macrophages Prime Naïve T Cells to Become Effector T Cells in β2m Deficient Mice

CD45.1 congenic P-14 T cells were CFSE-labeled and adoptively transferred into β2m Deficient hosts. One day later, mice were immunized SQ with gp33-pulsed DC (10 μg/ml) or MΦ (100 μg/ml). The draining lymph node was removed 4 days later and stained for CD8 and CD45.1. The data are displayed as CFSE content of the live, $CD45.1^+$ and $CD8^+$ producing IFN-γ. The numbers in the left corner represents the percentage of transgenic T cell producing IFN-γ. This experiment is representative of 3 experiments with a total n=10 mice/group.

Figure 23: Macrophages Prime Naïve T Cells to Become Effector T Cells in β2m Mice

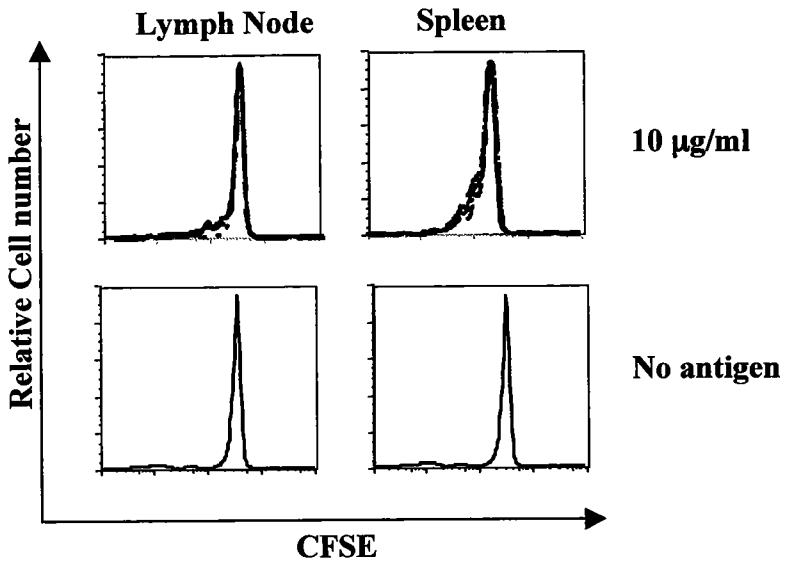


Since a small amount of priming was seen when T cells pulsed with gp33 were used as APCs in wild type mice (Fig. 13), we repeated these experiments in β2-microglobulin knockout mice. T cells were unable to stimulate naïve P-14 T cells in the class I-deficient mice (Fig. 24), indicating that the limited proliferation of transgenic T cells in wild type mice stimulated by peptide-pulsed T cells, was most likely due to cross-presentation. Taken together these experiments clearly indicate that MΦs can directly prime naive T cells. Once primed these primed T cells go on to gain effector function as indicated by cytolytic activity and IFN-γ secretion. In addition, T cells primed by both MΦs and DCs generate a pool of memory T cells. These memory T cells are able to secrete IFN-γ in an intracellular cytokine assay.

Figure 24: Most Of The Priming Observed When T Cell APCs Are Given IV Is By Cross-Presentation.

CD45.1 congenic P-14 T cells were CFSE-labeled and adoptively transferred into β2m Deficient hosts. One day later, mice were immunized IV with OT-I/Rag $^{-/-}$ T cells, DCs or MΦσ pulsed with 10 μg/ml or 100 μg/ml of gp33. The draining lymph node was removed 3 days later and stained for CD8 and CD45.1. The data are gated on live, CD45.1^{+} and CD8^{+} T cells and is displayed as CFSE content of transferred cells. The different color lines represent different mice in the same experiment. This experiment was repeated 2 times with a total n=8 mice/group.

Figure 24: All Priming by T Cells When Injected Intravenously is Cross Presentation



Chapter V: Macrophages And Dendritic Cells Prime Naïve $CD4^+$ T Cells *In Vivo*

It is known that the requirement for the priming of $CD4^+$ and CD8 T^+ cells and their dependence on costimulatory signals can be different. In light of these facts, we decided to perform a limited set of experiments to test the hypothesis that bone marrow-derived MΦs could prime naive $CD4^+$ T cells. To test this hypothesis, we took advantage of the DO11.10 TCR transgenic mice. These mice have $CD4^+$ T cells specific for the ovalbumin peptide 329-339 (ISQAVHAAHAEINEAGR) bound to the MHC II, IA^d molecules. An additional benefit to using this mouse is the existence of a clonotypic antibody anti-KJ1–26 allowing for easy tracking of antigen specific T cells. In these Class II experiments we hoped to answer the same kind of questions addressed in the Class I system. Can the MΦs prime naive T cells? If so, do the T cells gain effector function?

Results

Macrophages Prime Naive $CD4^+$ T Cells When Administered Subcutaneously

In contrast to what was seen in the class I system, MΦs were only weakly able to induce proliferation of naive T cells. For these experiments, lymph node T cells were isolated from DO11.10 transgenic animals and labeled with CFSE. $5x10^6$ transgenic T cells were transferred into BALB/c hosts and one day later the animals were immunized with $0.5x10^6$ peptide-coated APCs. On day 4, the draining lymph node was removed. Lymph node cells were stained with the clonotypic antibody and anti-CD4 and analyzed for proliferation by FACS. Figure 25 and 26 and shows the results of typical experiments.

Figure 25: Titration of Peptide Needed for Priming of DO11.10 T Cells by Dendritic Cells.

$5x10^6$ CFSE labeled $CD4^+$ T cells were transferred in BALB/c hosts. One day later, the mice were immunized with $0.5x10^6$ DCs pulsed with various concentration of $OVA_{323-339}$ SC. On day 4, draining lymph nodes were removed and stained with the clonotypic antibody KJ1–26 and anti-CD4 and analyzed by FACS. Data are shown as CFSE content of the transgenic T cells. This experiment was repeated 6 times with a total n=12 mice/group.

Figure 25: Titration of Peptide Needed for Priming of DO11.10 T Cells by DCs

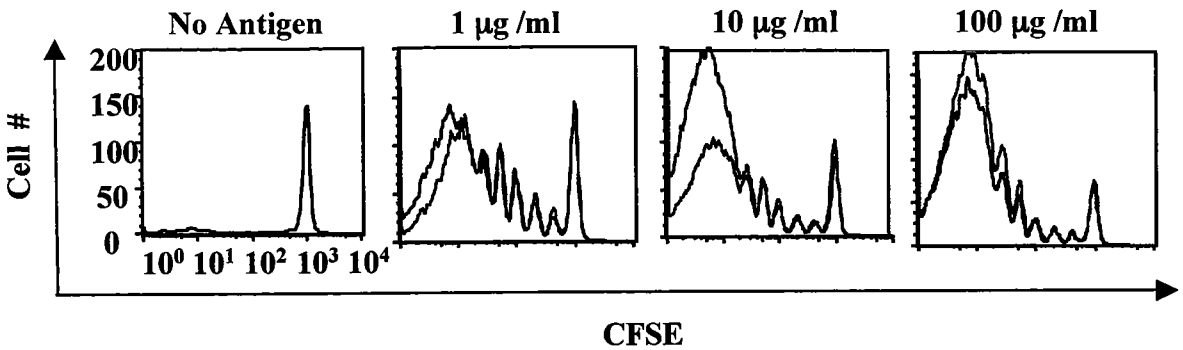


Figure 26: Titration of Peptide Needed for Priming of DO11.10 T Cells by Macrophages.

A. $5x10^6$ CFSE labeled $CD4^+$ T cells were transferred into BALB/c hosts. One day later, the mice were immunized with $0.5x10^6$ MΦs pulsed with 10 and 100 μg/ml OVA $_{323-339}$ SC. On day 4, draining lymph nodes were removed and stained with the clonotypic antibody KJ1–26 and anti-CD4 and analyzed by FACS. The different color lines represent individual animals in the same group. Data are shown as CFSE content of the transgenic T cells. This experiment was repeated 3 times with a total n=6 mice/group.

B. $5x10^6$ CFSE labeled $CD4^+$ T cells were transferred into BALB/c hosts. One day later, the mice were immunized with $0.5x10^6$ MΦs pulsed with 10 and 100 μg/ml of OVA $_{323-339}$ SC. On day 4, draining lymph nodes were removed and stained with the clonotypic antibody KJ1–26 and anti-CD4 and analyzed by FACS. The different color lines represent individual animals in the same group. Data are shown as CFSE content of the transgenic T cells. This experiment was repeated 3 times with a total n=6 mice/group.

Figure 26A: Titration of Peptide Needed for Priming of DO11.10 T Cells by MΦs

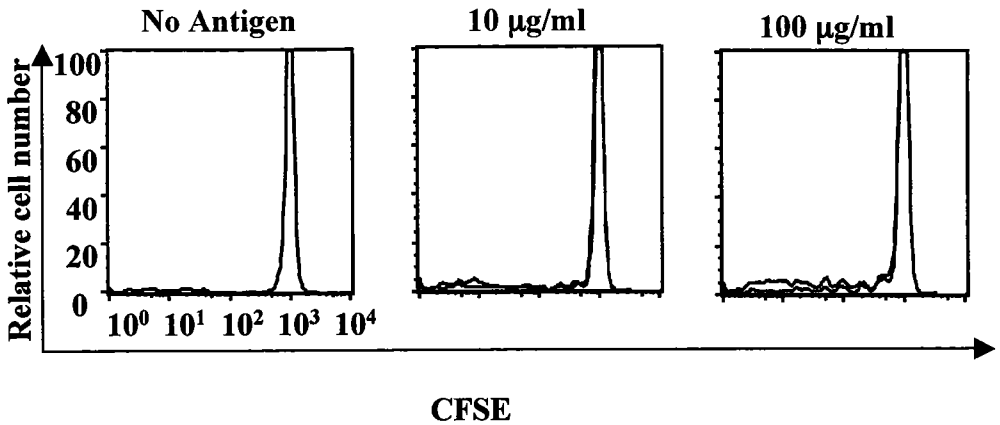
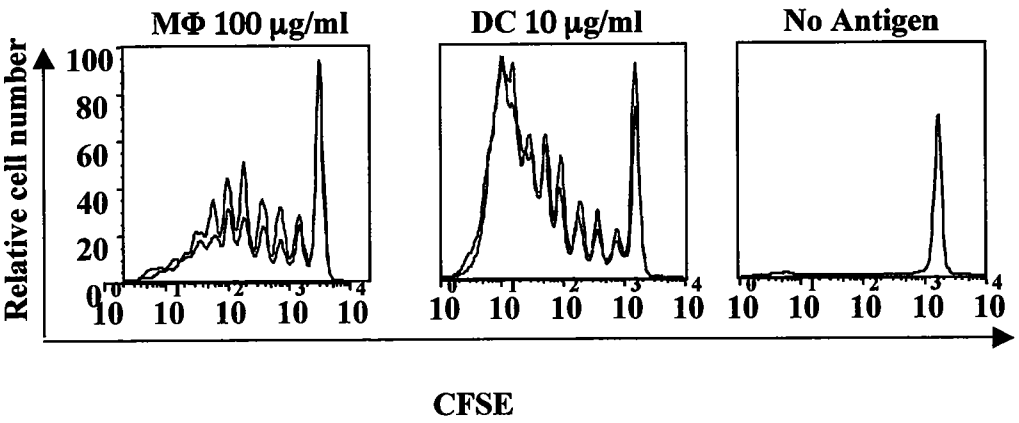


Figure 26B: Titration of Peptide Needed for Priming of DO11.10 T Cells by MΦs



Stimulation of the transgenic T cells with DCs pulsed with as little as 1 μg/ml of cognate peptide led to significant proliferation of the DO11.10 T cells (Fig.25). In contrast, Fig 26 panel A demonstrated that MΦσ are much poorer stimulators of $CD4^+$ T cells and require up to 100 μg/ml of the same peptide. In later experiments stimulation with MΦs led to better stimulation than in earlier experiments, but never to the same level as the DCs (panel B).

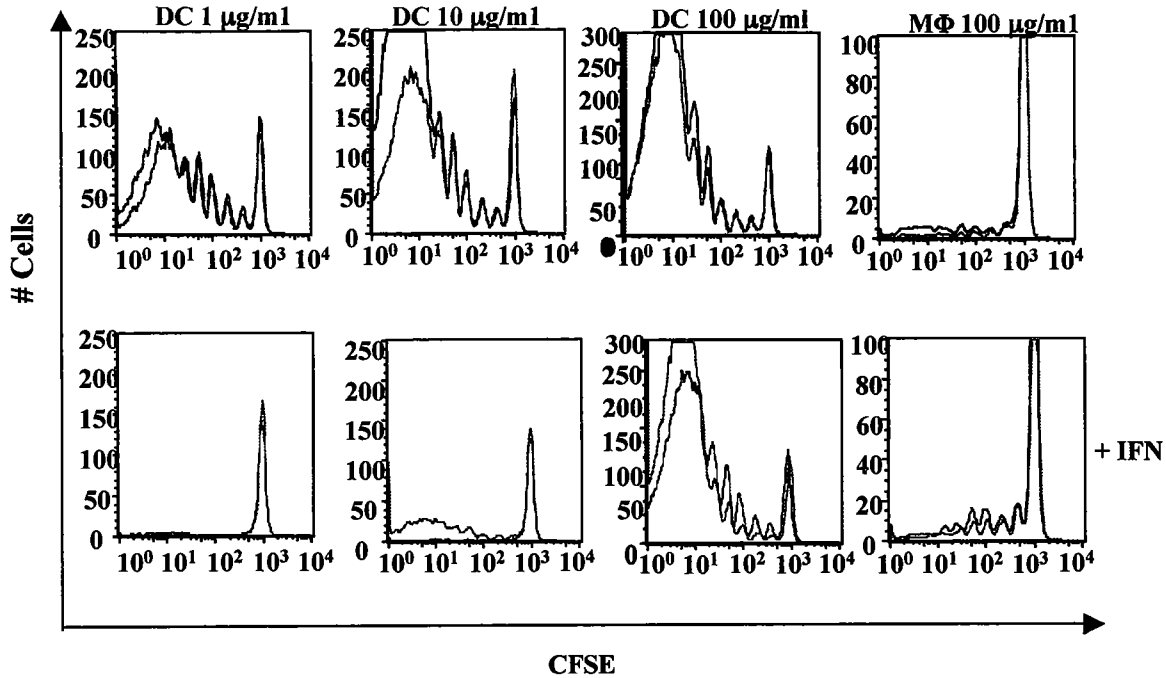
The Effect Of IFN-γ Stimulation On The Ability Antigen Presenting Ability To $CD4^+$ T Cells

Since DCs express higher levels of MHC class II molecules than MΦs (Fig. 1), we thought this might account for the lesser stimulatory ability of the MΦs. In order to up-regulate the expression of class II on the surface of the APCs, we stimulated the cells with IFN-γ. Stimulation of APC with IFN-γ is known to up-regulate various molecules involved in antigen processing and presentation including MHC I and MHC II levels (Kuby, 1997). The results of animals immunized with APCs stimulated with IFN-γ are shown in Fig. 27. Much to our surprise, stimulation of DCs with IFN-γ led to diminished ability to stimulate the $CD4^+$ T cells in our system. This reduction in priming ability was more strongly observed when DCs pulsed with lower peptide concentrations were used as APCs. We did not investigate the basis for this negative effect of IFN-γ stimulation.

Figure 27: The Effect of IFN-γ on the Ability of APCs to Stimulate $CD4^+$ T cells.

$5x10^6$ CFSE labeled CD4+ T cells were transferred in BALB/c hosts. Half of the MΦs and DCs were cultured for the last 48 hours in either IFN-γ or media. One day after T cell transfer, the mice were immunized with $0.5x10^6$ $OVA_{323-339}$ pulsed APC treated and untreated with IFN-γ. On day 4, draining lymph nodes were removed and stained with the clonotypic antibody KJ1–26 and anti-CD4 and analyzed by FACS. The different color lines represent individual animals in the same group. Data are shown as CFSE content of the transgenic T cells. This experiment was repeated 3 times with a total n=6 mice/group.

Figure 27: The Effect of IFN–γ on the Ability of APCs to Stimulate CD4$^+$ T cells



Cytokine Production Of The DO11.10 T Cells Primed In Vivo

Next we examined the ability of the T cells primed by the two APCs to differentiate into effectors. Several studies have previously shown that priming with different APCs can lead to a skewing of the Th1, Th2 profiles of the cells (Croft et al., 1992; Desmedt et al., 1998; Duncan and Swain, 1994). Another possibility was that the T cells were stimulated to enter division, but did not differentiate into effector cells. The results from a representative experiment can be seen in Table 6. The *in vivo* priming of DO11.10 T cells with DCs led to populations of cells producing all the cytokines examined. In contrast and surprisingly, priming by MΦs led to the T cells producing only IL-2. This experiment was repeated 4 times and the same trend was always observed. We found these results quite intriguing because the T cells stimulated by MΦs didn't display a Th0, Th1or Th2 phenotype. Since anergic cells are unable to produce IL-2 these data suggest these T cells are actually activated. We are currently repeating these experiments in a second transgenic mouse model whose T cells see the same peptide presented on IA^b, OT-II mice. These mice should allow us to evaluate the role of host APCs in this system as well as allow us to evaluate whether or not memory cells are generated in this system.

Table 6: Percentage Of D011.10 T Cells Secreting Cytokines.

$5x10^6$ CFSE labeled $CD4^+$ T cells were transferred in BALB/c hosts. One day later, the mice were immunized with $0.5x10^6$ MΦs or dendritic pulsed with various concentration of OVA $_{323-339}$ SC. On day 4, draining lymph nodes were removed incubated with 10 μg/ml of OVA $_{323-339}$ for 5 hours in the presence of Brefeldin A The samples were then stained with the clonotypic antibody KJ1–26 and anti-CD4 and analyzed by FACS. Data are shown as percent of $KJ1–26^+$ cells producing the various cytokines. Background staining with isotype control IgG was always less than 0.4%. The experiment was repeated 4 times with a total n=6 mice/group.

Table 6: % D011.10 T Cells Secreting Cytokines

Cytokine	No antigen	DC 1 μg/ml	DC 10 μg/ml	MΦ 10 μg/ml	MΦ 100 μg/ml
IL-2	0.6	21.8	14.9	2.14	3.77
GM-CSF	1.09	5.06	6.82	1.12	1.06
IFN-γ	0.6	5.59	2.38	1.13	1.11
IL-4	0.5	1.51	3.23	0.49	0.51
TNF	1.2	15.0	9.35	1.62	1.53

Chapter VI: How Do $CD8^+$ T Cells Eliminate The Antigen Presenting Cell?

In addition to studying how APCs stimulated T cells, we were also interested in the reciprocal question of how T cells influenced the APCs. At the time I initiated experiments to explore this question, little was known about the fate of the APCs. Ingulli *et al.* had reported an interesting observation. Using an adoptive transfer model, these authors imaged the interaction of peptide pulsed DCs with antigen specific T cells in the draining lymph node by fluorescent microscopy. Using $CD4^+$ T cells and splenic DCs labeled with fluorescent dyes, they demonstrated that the maximum numbers of transferred DCs were present at 24 hours. Another observation made by these authors was that in the animals that received DCs pulsed with the transgenic T cells cognate peptide, there were no APCs in the lymph node by the 48 hour time point, whereas, unpulsed DCs were still present in LNs after 72- hours. The authors concluded that the antigen presentation event must be a terminal one for the antigen-presenting cell. Since the T cell used in this study were $CD4^+$ T cells it was speculated that the killing of the APC occurred through a Fas-FasL mechanism, however no experiments were performed to investigate this idea (Ingulli et al., 1997). I decided to explore whether a similar phenomenon occurred when DCs presented antigen to $CD8^+$ T cells and to investigate whether known cytotoxic mechanisms used by $CD8^+$T cells might be involved in this process. While my experiments were in progress, a number of papers were published on this subject (discussed below)

Results

Possible Role Of Fas-FasL Interactions In The Antigen Specific Elimination Of DCs

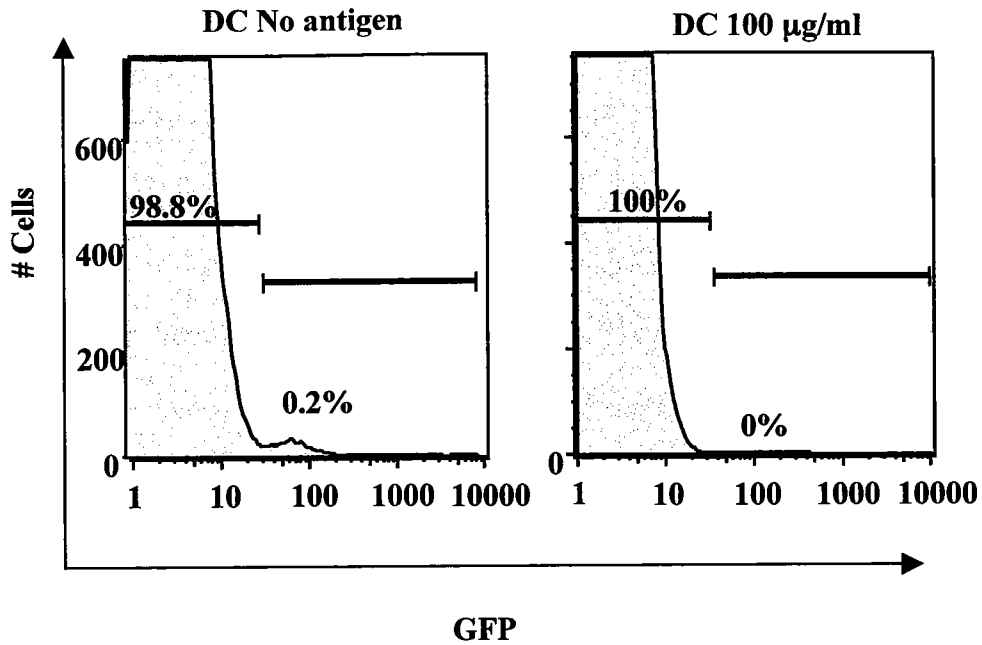
OT-I transgenic T cells were transferred intravenously into wild type hosts. The next day DCs from either Fas-deficient or wild type mice pulsed with various peptide concentrations and CFSE labeled were injected into the hind footpad. The draining lymph node was removed 48 or 72 hours later. The popliteal lymph nodes were digested with collagenase and DNase to make single cell suspensions. The samples were then run on a FACS caliber counting 500,000 large forward side scatter cells as previously described by Hermans *et al.* (Hermans et al., 2000). This FACS method was validated using DCs from transgenic mouse expressing GFP under the control of the class I promoter. Figure 28 shows the liberation of the GFP^+ DC as well as the disappearance of the DCs at high antigen concentrations. This figure demonstrates that we are able to liberate the DCs and accurately visualize them by FACS analysis. In addition, Fig. 28 shows that the elimination of the peptide-pulsed DCs is dependant on the presence of antigen specific T cells.

Although there are many molecules involved in inducing death in target cells, we chose to begin this study looking at whether or not Fas-FasL interactions played a role in the elimination of the APC after presenting to $CD8^+$ T cells. Fas is a member of the TNF superfamily and upon ligation the Fas expressing cell will undergo an apoptotic program. Fas is expressed on a variety of cell types including T cells, B cells, MΦs and DCs, however, the expression of FasL is more tightly regulated and is restricted to MΦs, DCs

Figure 28: Assay For Disappearance For Pulsed Dendritic Cells.

$2.5x10^6$ CFSE labeled OT-I/GFP^+ transgenic T cells were adoptively transferred into C57BL/6 mice. One day later, animals were immunized SC with unpulsed or OVA-p pulsed (100 μg/ml) DCs. Seventy-two hours later, draining lymph nodes were removed and digested with collagenase and DNase. The samples were then analyzed by FACS. 500,000 large forward side scatter events were collected. The data are shown as the percentage of large cells expressing GFP.

Figure 28: Assay For Disappearance for Pulsed Dendritic Cells



and activated T cells. Fas plays an important role in $CD4^+$ T cell homeostasis and a lesser role in $CD8^+$ T cells homeostasis. $CD4^+$ and $CD8^+$ T cells can also both use Fas-FasL as a method of lysing target cells. Expression of Fas by activated T cells and has been shown to lead to APC death of DCs, MΦs and B cells *in vitro* (Ashany et al., 1995; Oddo et al., 1998; Sad et al., 1996; Stenger et al., 1997).

In order to examine the possible role of Fas in the observed DC disappearance, DC were grown from the BM of Fas-deficient mice (Lpr) and transferred into wild type hosts that had received wild type OT-I transgenic T cells the previous day. Data from a representative experiment from the original set of experiments can be seen in Figure 29. The Y-axis indicates percentage of cells disappearing and the x-axis shows APCs injected into the footpads of the mice. At the limiting antigen concentration of 100 ng/ml, 80% of wild type DCs are eliminated by 48-hours, whereas all of the Fas-deficient APCs still remain in the node. Pulsing the DCs with 1 μg/ml of SIINFEKL led to almost total elimination of the wild type cells and about a 50% disappearance of the Fas deficient cells. In contrast, at the highest antigen concentration tested, 10 μg/ml, Fas plays no role in the antigen specific DCs disappearance observed. In later experiments, even a small role for Fas at lower antigen concentrations could not be seen. Figure 30 shows an example from this set of experiments. In this set of experiments, the antigen specific T cells were able to eliminate the Fas deficient DCs and well as the wild type DCs, even at low peptide concentrations. From these experiments it was concluded that if Fas-FasL interactions play a role in the disappearance of the DCs, its role is minor.

Figure 29: Fas Deficient DCs Have Increased Longevity.

$2.5x10^6$ CFSE labeled OT-I transgenic T cells were adoptively transferred into C57BL/6 mice. One day later, animals were immunized SC with CFSE labeled wild type or Fas deficient DCs. The DCs were pulsed with various concentrations of OVA-p or incubated with media alone as a control. Forty-hours later, draining lymph nodes were removed and digested with collagenase and DNase. The samples were then analyzed by FACS. 500,000 large forward side scatter events were collected. The animals that received DCs from C57BL/6 mice are shown in dark blue and the animals that received DCs from Fas deficient mice are shown in the turquoise. The data are shown as the percentage of large cells expressing CFSE. This experiment was repeated 3 times with a total n=6 mice/group.

Figure 29 : Fas Deficient DCs Have Increased Longevity

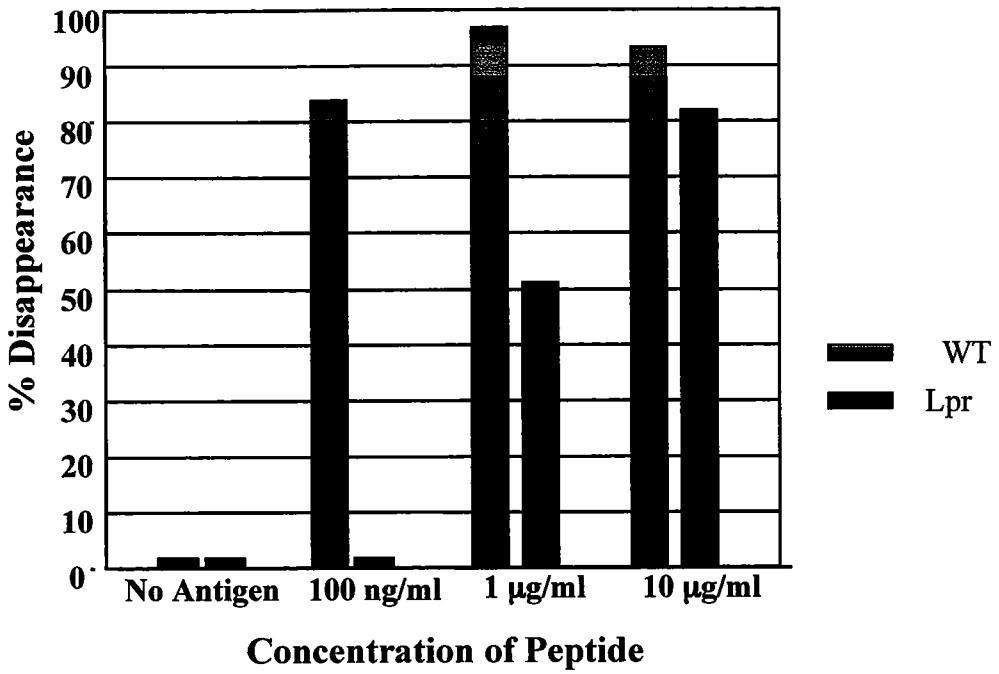
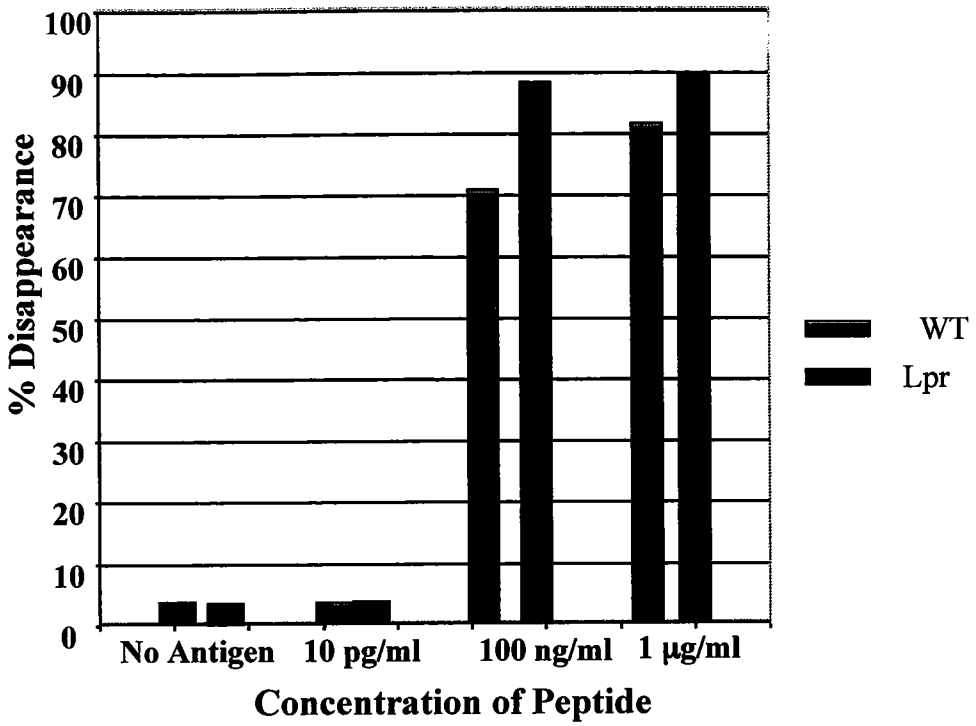


Figure 30: Fas Has No Effect On DC Longevity.

$2.5x10^6$ CFSE labeled OT-I transgenic T cells were adoptively transferred into C57BL/6 mice. One day later, animals were immunized SC with CFSE labeled wild type or Fas deficient DCs. The labeled DCs were pulsed with various concentrations of OVA-p or incubated with media alone as a control. Forty-hours later, draining lymph nodes were removed and digested with collagenase and DNase. The samples were then analyzed by FACS. 500,000 large forward side scatter events were collected. The animals that received DCs from C57BL/6 mice are shown in dark blue and the animals that received DCs from Fas deficient mice are shown in the turquoise. The data are shown as the percentage of large cells expressing CFSE. This experiment was repeated 6 times with a total n=12 mice/group.

Figure 30: Fas Has No Effect On DC Longevity



This conclusion is consistent with that of other labs that performed similar experiments published after we finished these studies (Loyer et al., 1999; Ludewig et al., 2001).

Possible Role Perforin In The Antigen Specific Elimination Of DCs

Although $CD4^+$ T cells are restricted to killing by Fas, $CD8^+$ T cells can also use perforin to kill target cells. Once $CD8^+$ T cells become activated they produce perforin and granzymes and store them in granules. The granzymes are serpins and are actually the death-inducing molecules. Once a CTL binds to its target, perforin forms pores through which the granzymes are exocytosed in to the target cell. The target cell will then undergo apoptosis. Perforin has been shown to be necessary for clearance of LCMV and *Listeria* as well as elimination of tumor cells. On the other hand, perforin is dispensable for protection to vesticular stomatitis virus and vaccinia virus (Kagi et al., 1996).

Since activated $CD8^+$ T cells become CTL effectors, it is possible that the OT-I T cells eliminate the peptide-pulsed DC through expression of the effector molecule perforin. To test this hypothesis, we bred the OT-I mice to perforin knockout mice and used these as effector T cells in our adoptive transfer model. Figure 31 shows the results of a typical experiment. The use of T cells deficient in perforin had no effect on the magnitude of DC disappearance when assayed on day 2 at any of the peptide concentration examined. One of the benefits of this adoptive transfer system is that T cells and APCs with different deficiencies could be used in the same experiments. We took advantage of this fact and used perforin deficient OT-I T cells and Fas deficient DCs in the same mice.

In this case, we consistently saw a change in the percentage of DCs disappearing. At high peptide concentrations, the absence of both Fas and perforin led to a 50% decrease in the ability of the T cells to eliminate the peptide-pulsed DCs. It is important to note that half of the DCs still did disappear, suggesting that multiply pathways regulate this important function (Fig. 31). These results seen using perforin deficient T cells, were confirmed in a study by Ludewig *et al.* These authors showed that although perforin played a role in the killing of peptide pulsed DCs in *vitro it* did not play a major role *in vivo.* (Ludewig et al., 2001).

In conclusion, it appears multiple death pathways are involved in the elimination of antigen presenting cells by primed $CD8^+$ T cells. In light of our early data showing that Fas possibly played a role at lower peptide concentrations as well as the results of combining Fas deficient DC with perforin deficient T cells, it is still possible Fas-FasL interactions play a small role in this phenomenon. These studies also suggest that perforin can also play a partial, but redundant role. What has become clear is the existence of other unknown players in this pathway.

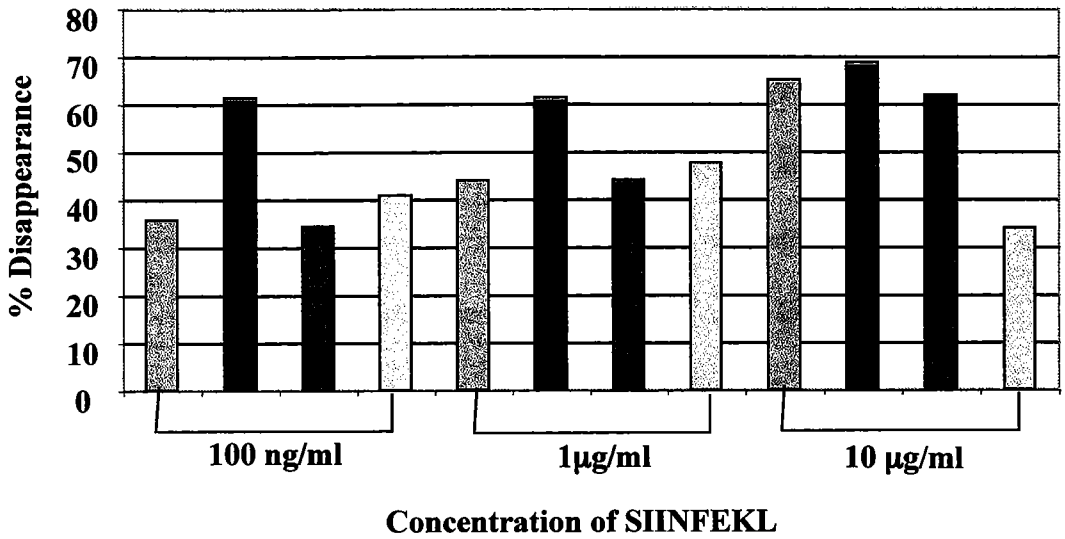
Possible Role Of Fas-FasL Interactions In The Expansion of Antigen Specific $CD8^+$ T cells

One possible trivial explanation for why we not seeing disappearance may be because the T cells were not adequately stimulated and therefore did not acquire effector functions. In order to rule out this possibility, these experiments were repeated using congenic T cells. The use of the congenic marker, in this case CD90.1, allowed us to quantify T cell expansion in the same experiments. These experiment were performed

Figure 31: The Combined Effect of DC Fas Deficiency and T Cell Perforin Deficiency.

$2.5x10^6$ CFSE labeled OT-I or OT-I/Perforin $^{-/-}$ transgenic T cells were adoptively transferred into C57BL/6 mice. One day later, animals were immunized SC with CFSE labeled wild type or Fas deficient DCs. The labeled DCs were pulsed with various concentrations of OVA-p or incubated with media alone as a control. Forty-hours later, draining lymph nodes were removed and digested with collagenase and DNAse. The samples were then analyzed by FACS. 500,000 large forward side scatter events were collected. The data are shown as the percentage of large cells expressing CFSE. This experiment was repeated 3 times with a total n=6 mice/group and the same trend was always observed.

Figure 31: : The Combined Effect of DC Fas Deficiency and T Cell Perforin Deficiency



exactly as the other disappearance experiments; except some animals were harvested at later time points and the lymph node cells were stained with APC-conjugated antibody against CD90.1 to quantify the expansion of antigen specific T cells. The results of this experiment were intriguing. We had hypothesized that if we could extend the life of the presenting APC, it would lead to a greater degree of T cell expansion. Figure 32 a shows the results of these experiments. We sacrificed mice at day 4, 5,7 and 10 days after DC transfer. This figure shows days post DC transfer on the X-axis and % $CD8^+$ T cell expressing the transgenic marker on the Y-axis. Figure 32 demonstrates that when DCs are pulsed with equal amounts of peptides, Fas-deficient T cells leads to a lesser expansion of the antigen specific T cells. It was possible that the T cells stimulated by the Fas-deficient DCs proliferated earlier than those stimulated with wild type DCs. To rule out this possibility, we harvested lymph nodes from mice 2, 3 and 4 days after DC transfer. Once again, we observed lesser expansion of the OT-I transgenic T cells when Fas-deficient DCs were used as stimulators as compared to wild type DCs pulsed with the same amount of peptide (Fig 32b). This inability of Fas-deficient DCs to stimulate transgenic T cells to the same extent as wild type DC supports a role for FasL as a costimulatory molecule. In fact after we finished this study, two studies were published demonstrating a role for Fas-FasL interaction as a costimulatory signal for $CD8^+$ T cells, but not $CD4^+$ T cells (Suzuki and Fink, 2000; Suzuki et al., 2000). Using T cells from FasL-deficient mice, these authors demonstrated the ability of FasL engagement to

Figure 32: Accumulation Of OVA-P Specific T Cells By Fas Deficient Dendritic Cells.

A. $2.5x10^6$ $CD90.1^+$ OT-I transgenic T cells were adoptively transferred into C57BL/6 mice. One day later, animals were immunized SC with peptide-pulsed wild type or Fas deficient DCs. The DCs were pulsed with various concentrations of OVA-p or incubated with media alone as a control. Draining lymph nodes were removed 4,5,7 and 10 days later and digested with collagenase and DNase. The samples were then analyzed by FACS. 500,000 large forward side scatter events were collected. The animals that received DCs from C57BL/6 mice pulsed with 100 ng/ml are shown in dark blue and the mice pulsed with 10 ng/ml are shown in turquoise. DCs isolated from Fas deficient mice pulsed with 100 ng/ml are shown in red and the DCs isolated from Fas deficient mice pulsed with 10 ng/ml are shown in fuchsia. This experiment was repeated 3 times with a total n=3 mice/group.

B. The experiment was performed exactly as above except lymph nodes were removed after 2,3, and 4 days. Wild type DCs pulsed with various concentrations of OVA-p: no peptide (black), 10 pg/ml (orange), 100 ng/ml (blue) and 1 μg/ml (yellow). DCs from Fas deficient mice pulsed with various concentrations of OVA-p: no peptide (grey), 10 pg/ml (turquoise) and 100 ng/ml (lilac). This experiment was repeated 3 times with a total n=3 mice/group.

Figure 32A: Accumulation of OVA-p Specific T Cells by Fas Deficient Dendritic Cells

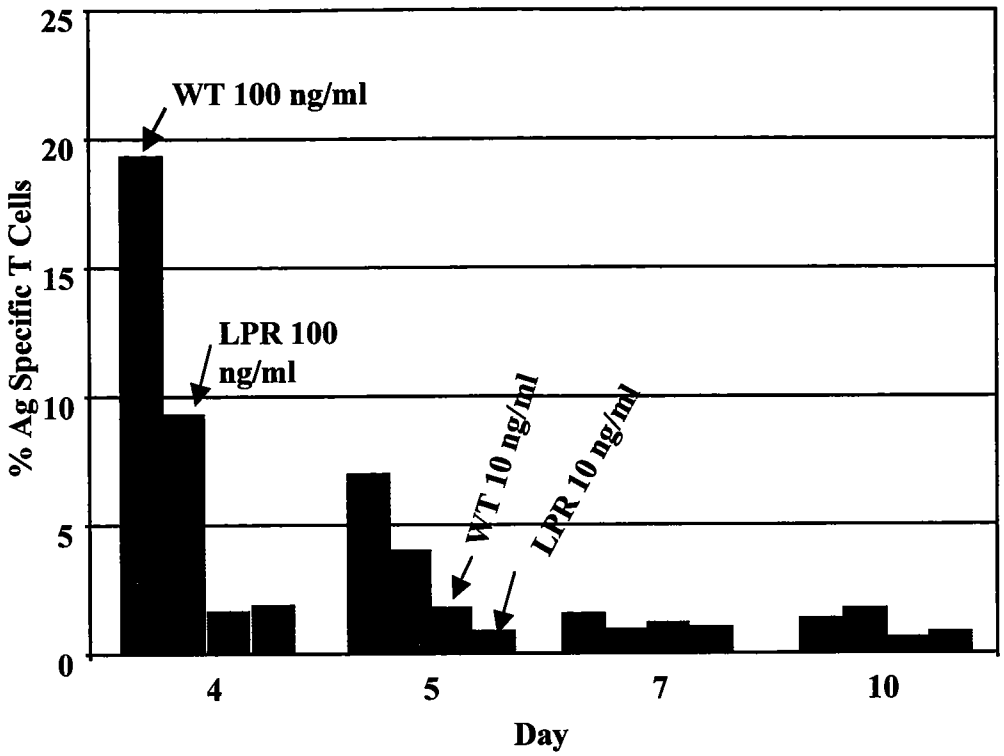
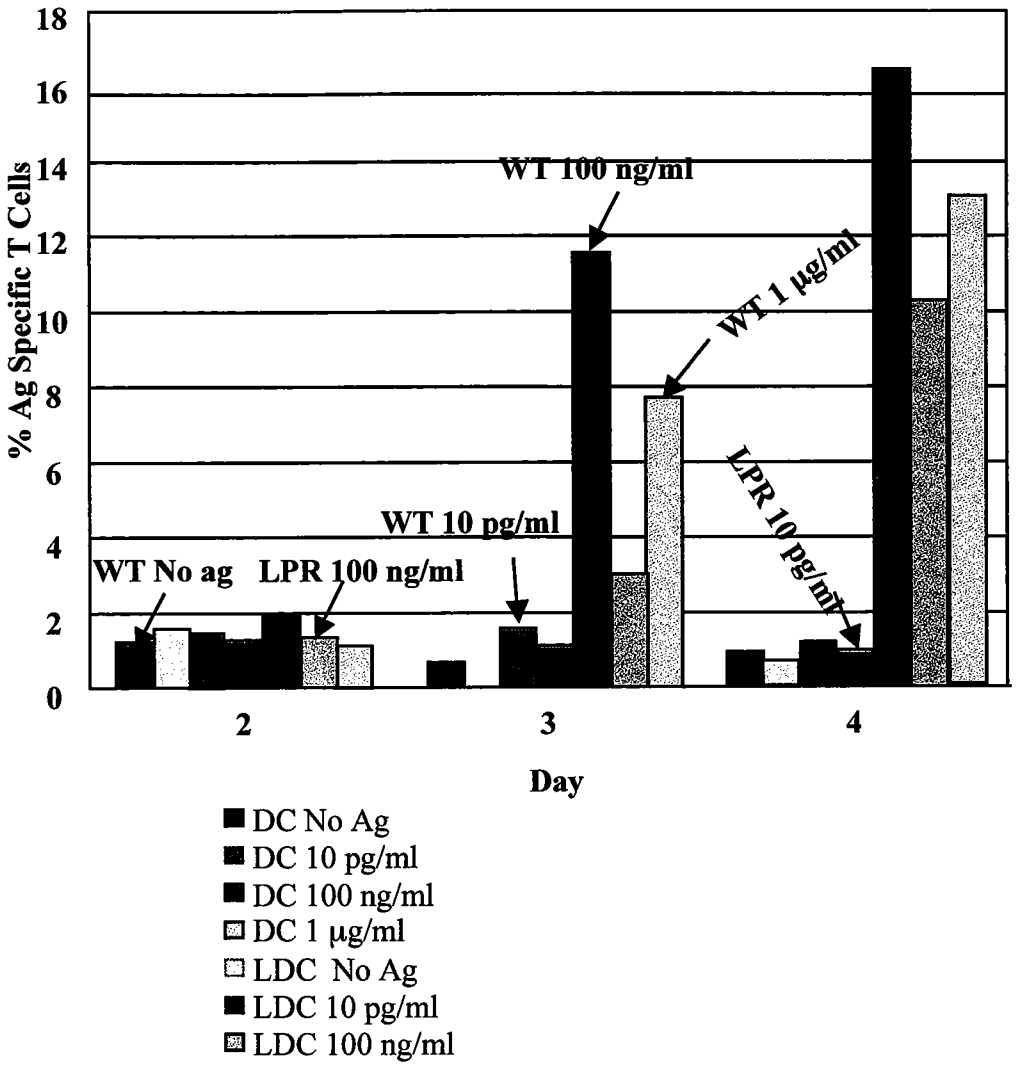


Figure 32B: Overall T Cell Accumulation With Fas Deficient DC Stimulation



increase maximal $CD8^+$T cells proliferation when the T cells were stimulated through their TCR. It role for FasL in increasing $CD4^+$T cell proliferation was also shown, but only when the ability of the $CD4^+$T to undergo Fas mediated death was blocked.

Chapter VII: Discussion

Priming Of Naive CD8$^+$ T Cells

Naïve CD8$^+$ T lymphocytes have more stringent requirements for activation than effector T cells and hybridomas. The issue of what APC initially stimulates these T cells in the initiation of immune responses is an important one. It is well established that DCs can stimulate primary immune responses and our data confirm this point. *In vitro* there is abundant evidence that MΦs acquire antigen and stimulate CD8$^+$ T cells (Apostolopoulos et al., 2000; Hosono and Katsura, 1982; Torpey et al., 1989; Wijburg et al., 1998). Our data show that MΦs can also stimulate CD8$^+$ T cells *in vivo* to proliferate, express effector functions and mature into memory cells. We have also shown that this stimulation is due to direct stimulation of T cells by MΦs and not cross-presentation by or contamination with small numbers of DCs. Based on these findings we conclude that MΦs can also directly prime naive T cell responses.

Our conclusion is surprising, because it has previously been argued that DCs are the only APC that initiate primary immune responses (Banchereau and Steinman, 1998; Jung et al., 2002; Norbury et al., 2002; Steinman et al., 1983) and that MΦs play no role in this process. However, the published evidence supporting this view is relatively limited. Much of the evidence comes from *in vitro* experiments. Steinman and coworkers tested various purified APCs in a mixed lymphocyte reaction and found that DCs were strong activators of T cells, whereas MΦs were poor stimulators of such responses (Steinman et al., 1983; Steinman and Witmer, 1978). Depleting DCs from splenocytes with an antibody virtually eliminated their ability to stimulate MLRs (Steinman et al., 1983; Steinman and Witmer, 1978). DCs were also able to stimulate

primary T cell responses to foreign antigen *in vitro* (Inaba et al., 1990; Inaba and Steinman, 1986). However, it is unclear whether the *in vitro* experiments accurately model the *in vivo* situation. Moreover, in other *in vitro* studies there is opposite data indicating that other APCs can stimulate primary $CD8^+$ T cell responses (Butz and Bevan, 1998).

Another approach to identify the type of APCs needed to initiate $CD8^+$ T cell responses has been to deplete different subsets of APCs *in vivo*. Agents such as silica or chlodronate-liposomes eliminate phagocytic cells *in vivo* and inhibit the generation of T cell responses (Ciavarra et al., 1997; Debrick et al., 1991; Nair et al., 1995; Wu et al., 1994). Such experiments were originally interpreted to demonstrate a role for MΦs in T cell priming. However, it was later appreciated that immature DCs are also phagocytic and likely to be eliminated by these treatments (Banchereau and Steinman, 1998). Although, one group tested their liposomes made from dichloromethylene diphosphonate and found no DC toxicity further supporting a role for MΦs in priming naive T cells in their vesticular stomatitis system (Ciavarra et al., 1997).

A more selective approach for depleting DCs used transgenic mice that express the receptor for Diphtheria toxin under the control of the CD11c promoter (a DC specific promoter). Administration of diphtheria toxin selectively eliminated CD11c-positive DCs in these transgenic mice. Using this approach, Jung *et al.* demonstrated that elimination of DCs by diphtheria toxin blocked the generation of CTLs to some antigens, e.g. *Listeria moncytogenes* and *Plasmodium yoelii* (Jung et al., 2002). This data are among the strongest to shown that DCs can play an essential role in stimulating T cell

responses at least for certain antigens. However, whether this is true for other antigens is unknown; *in vivo* some antigens may be preferentially presented on DCs while others are also presented on MΦs (see above). Also, a caveat in the diphtheria-toxin studies was the possibility that the ability of MΦs to stimulate T cells was impaired by the toxin, e.g. when MΦs ingested the toxin during phagocytosis of dying DCs.

In a set of experiments designed to visualize the APCs that interacted with naive $CD8^+$ T cells *in situ*, Norbury *et al.* infected mice with a vaccinia virus expressing EGFP and could detect infected (EGFP-expressing) DCs and MΦs *in situ*. When they transferred CFSE-dye-labeled virus-specific T cells into these same animals, they observed clustering of these $CD8^+$ T cells around EGFP-positive (infected) APC. Although MΦs made up > 60% of infected cells, clusters were observed only around the infected DCs. Although this was interpreted to show that only DCs were stimulating the T cells, T cell stimulation was actually being inferred. What was actually measured, T cell-APC clustering, is influenced by strength of adhesion, chemokines and potentially other factors. In fact, DCs can cause T cells to cluster under some circumstances, even in the absence of specific antigen (Inaba and Steinman, 1986). This analysis would also fail to detect single T cells that were stimulated and/or ones that had detached from an APC. Moreover, a substantial component of the T cell response to vaccinia occurs through cross-priming (Norbury et al., 2001; Ramirez and Sigal, 2002; Sigal et al., 1999) and the APCs involved in this process would not express EGFP from the vaccinia recombinants. Consistent with a cross-priming mechanism, T cells were observed to cluster with EGFP-negative cells and many of these latter cells lacked DC markers. These finding are also

limited to vaccinia virus. Finally, these experiments did not rule out the possibility of priming by the infected MΦs, nor did it show a correlation between the observed T cell clustering and the acquisition of T cell effector function (Norbury et al., 2002).

In summary, DCs can stimulate primary T cell responses and may be particularly potent in doing so. However, the evidence that they are the only cells that can prime responses and that MΦs lack this capability is relatively scarce. It is possible that DCs are the principal APCs for presenting some antigens (e.g. Listeria and Malaria). On the other hand, our data unambiguously demonstrate that MΦs can also stimulate naïve $CD8^+$ T cells *in vivo*. Our data additionally demonstrate that this observed presentation is direct presentation and not cross-presentation, since it occurs in two model systems where cross-presentation is not possible.

How do MΦs compare to DCs in stimulating T cell responses? *In vitro* DCs are reported to be 100-1000 times more potent APCs than MΦs. Interestingly, however, these two APCs can cross-present exogenous antigen with similar efficiency (Shen et al., 1997). Therefore, the difference in stimulating T cells *in vitro* may be due to lower levels of costimulatory molecules and/or MHC class II molecules on MΦs. However, the expression of both MHC and costimulatory molecules on MΦs can be increased upon activation of these cells thereby increasing their potency *in vivo*. *In vivo*, we also find differences in the potency of MΦs compared to DCs, although this appears to be most related to the ability of these cells to migrate to lymphoid tissue. When the MΦs are injected subcutaneously, it takes 10-fold more antigen to prime naive T cells compared to DCs; in contrast, when the APCs are injected via an intravenous route, they are of equal

potency at the same antigen concentration. Moreover, we have shown that quadrupling the number of MΦs injected subcutaneously results in the priming of equivalent responses. In fact, when we quantified the number of MΦs and DCs that migrated and compared the magnitude of T cell responses they stimulated, MΦs in the lymphoid tissues were actually more potent than DCs. However, such a comparison makes the assumption that the recovery of MΦs and DCs from lymphoid organs is similar when we dissociate the tissue to quantify APC migration; we don't know if this assumption is valid. In any case, we conclude that MΦs are bone fide stimulators of T cells.

An important implication of these findings is that the number of MΦs versus DCs present at a site of antigen deposition may influence which of these cells will contribute more to priming of $CD8^{+}$T cells. During inflammation, there is a large increase in the number of MΦs from recruitment and proliferation. Under these conditions, MΦs are much more numerous than DCs (Bryant and Ploegh, 2004; Croft et al., 1992; Meier et al., 2003). Therefore, MΦs could be the dominant APC in many situations, particularly infection. Since all of the experiments in this thesis used peptide pulsed cells, it is possible that different forms of antigen may be presented better by one cell type. In addition, it also possible that tissue or peritoneal MΦs could have different propensities for presenting different forms of antigen as well as varying abilities to present antigen.

Another interesting observation that might affect the potency of APCs, was the shorter half-life of SIINFEKL-MHC class I complexes on MΦs compared to DC. The peptide-complexes on immature DCs have a half life of about 3 days and those on mature DCs (CD80/CD86, class II high cells) are remarkably stable with a half life of >96 hours.

This observation suggests that once a DC matures, its peptide-MHC class I molecules are some how stabilized and/or their rate of turnover is decreased (Guermonprez et al., 2002; Rescigno et al., 1998 ; Zehn et al., 2004). Whether this difference in the half-life of MHC complexes on MΦs and DCs makes a difference in their ability to stimulate immunity *in vivo* is not yet clear. The long half-life of complexes on DCs may not lead to longer stimulation of T cells because the DCs are rapidly eliminated after presenting antigen to T cells (Hermans et al., 2000; Ingulli et al., 1997) (see also this thesis). On the other hand, it is possible that the shorter half-life of the MHC class I complex on MΦs may be offset by the continuous production of new peptides (in situations involving the processing from whole antigens).

Priming Of Naive CD4$^+$ T Cells

In vitro there is abundant evidence that MΦs acquire antigen and stimulate CD4$^+$ T cells (Askonas et al., 1968; Hsieh et al., 1993a; Hsieh et al., 1993b; Kahlert et al., 2000; Nair et al., 1995; Unanue and Askonas, 1968; von Delwig et al., 2002). Moreover, it is also well established that *in vitro* MΦs can take up and present particulate and cell-associated antigens on MHC class I and class II molecules (Brunt et al., 1990; Kovacsovics-Bankowski et al., 1993; Kovacsovics-Bankowski and Rock, 1994; Rock et al., 1993). Similarly, MΦs have been isolated from animals injected with viruses (Hamilton-Easton and Eichelberger, 1995; Usherwood et al., 1999) or soluble antigen (Grant and Rock, 1992) and shown to present antigen to CD4$^+$ T cells *ex vivo*. Interestingly, in some studies DCs are the only antigen-bearing APCs isolated from antigen-injected animals (den Haan et al., 2000). *In vivo*, the presentation of antigen by

MΦs to T cells is clearly important for type IV hypersensitivity responses and clearance of intracellular pathogens. In all of these situations MΦs are stimulating previously activated (effector) T cells or hybridomas.

The identity of the APC responsible for $CD4^+$ T cell priming *in vivo* is still an open question. Some studies have shown that the APC responsible depends on the form of antigen. Using I-E transgenic mice, Constant *et al.* demonstrated that although presentation of peptide antigen required DCs, presentation of whole protein antigen required the presence of B cells (Constant et al., 1995a). Many other studies have demonstrated a role for DCs in the priming of naive $CD4^+$ T cells (Erb et al., 1996; Flohe et al., 1998; Inaba et al., 1993; Ingulli et al., 1997; Ingulli et al., 2002; Steinman et al., 1983; Steinman and Witmer, 1978). It is also possible that the route of immunization as well as the form of antigen play a role in which APC can prime $CD4^+$ T cells populations. Inguilli *et al.* demonstrated that although B cells, MΦs, $CD11b^+$ and $CD8^+$ DCs all take up soluble OVA delivered subcutaneously, only the CD11b DC population was able to prime naive $CD4^+$ T cells (Ingulli et al., 2002). Others studies have shown that MΦs pulsed with peptide *ex vivo* can stimulate $CD4^+$ T cells (Croft et al., 1992; Duncan and Swain, 1994).

Since we were using $CD4^+$ T cell proliferation as a marker of T cell activation, and since proliferation has been shown not to always lead to effector function, we wanted to determine whether the MΦ primed $CD4^+$ T cells developed into effector T cells (Adler et al., 2000). Adler *et al.* demonstrated, using mice that express high and low levels of hemagglutinin (HA) on their peripheral cells, that antigen dose is not all that determines

tolerance versus activation of T cells. They went on to demonstrate that even though the level of HA expression varied 1000 fold in these 2 types of mice, the T cells became tolerant to the self-antigen in both systems. In addition, the transgenic T cells from both the high and low expressers proliferated for the same number of divisions and at the same rate when they encountered viral expressed HA. It can be concluded from this study that insufficient stimulation of T cells because of low peptide-MHC complexes is not what leads to tolerance induction in this system (Adler et al., 2000).

A role for the type of APC in the skewing of $CD4^+$ helper cell development has also been demonstrated (Constant et al., 2002; Desmedt et al., 1998; Duncan and Swain, 1994; Glimcher and Murphy, 2000; Rissoan et al., 1999). Desmedt *et al.* have shown that MΦs pulsed with peptide *ex vivo* can influence whether or not the $CD4^+$ T cells differentiate into Th0, Th1 or Th2 cells. These authors went on to show that stimulation of $CD4^+$ T cells with MΦs strongly favored Th1 cell development. In addition, it was also shown that stimulation of $CD4^+$ T cells with MΦs could also suppress already developing Th2 cell responses (Desmedt et al., 1998). Studies by Maldonado-Lopez demonstrated the ability of different DC populations to influence the ability of $CD4^+$ T cells to develop into Th1 or Th2 cells *in vivo*. Where as immunization of mice with antigen-pulsed $CD8^+$ DC led to the T cells differentiating into Th1 type cells, immunization of mice with antigen-pulsed $CD8^-$ DC led to the development of T cells with a Th2 phenotype (Maldonado-Lopez et al., 1999). This group further demonstrated that the ability of the DCs to skew T cells development depended upon the ability to secrete certain cytokines. In addition, the ability of $CD8\alpha^+$ DCs produce IL-12 and

preferentially stimulate $CD4^+$ T cells to become Th1 cells was also demonstrated (Maldonado-Lopez et al., 2001). It has also been demonstrated that myeloid DCs lead to the development of Th2 cells possibly by the secretion of IL-6 (Diehl and Rincon, 2002). There has also been data suggesting a role for antigen dose and structure. Experiments involving the use of altered peptide ligands have demonstrated a role for strength of TCR signal in determining lineage commitment (Glimcher and Murphy, 2000).

In light of the above-mentioned studies, we wanted to investigate whether the priming of the naive DO11.10 T cells by the MΦs allowed the $CD4^+$ T cells to express effector function. In addition, we wanted to evaluate the ability of DCs and MΦs to induce the secretion of various effector cytokines. We chose to use intracellular cytokine staining as the method of detection for the cytokines. The cytokines used to identify Th profile were: IL-2 and GM-CSF for Th0 cells, IFN-γ and TNF-α for Th1, and IL-4 for Th2. Using this model, we analyzed whether or not the stimulated T cells underwent multiple rounds of cell division and gained effector function. We found that MΦs could indeed stimulate naive $CD4^+$ T cells to proliferate and secrete IL-2. In addition it was demonstrated that DCs also primed naive T cells to undergo multiple rounds of cell division. In contrast to MΦs stimulated T cells, the DC primed T cells were able to secrete IFN-γ, TNF-α, IL-2, IL-4 and GM-CSF. These data suggest that DCs stimulate $CD4^+$ T cells to gain a greater range of effector functions than MΦs.

The mechanisms that underlie these differences will be of interest to investigate in future studies. In addition, although, the finding that MΦs can initiate $CD4^+$ T cell responses is an important one there are still many unanswered questions in the $CD4^+$ T

cell system. Do the stimulated T cells become memory cells? Is the adoptively transferred cell the APC responsible for the priming or is the peptide being transferred to host APCs? If the T cells become memory cells, are these cells still functional? And finally, would the macrophage primed $CD4^+$T cells be able to offer protection form an antigenic challenge?

Issues Related To Priming Of Both $CD4^+$ And $CD8^+$ T Cells

Monocytes can differentiate into both MΦs and DCs. It has been reported that monocytes can capture antigen in peripheral tissue and subsequently convert into DCs once they migrate into the lymph node (Randolph et al., 1999). It has also been reported that the fate of differentiating monocytes can be determined by migration pattern. Using a transendothelial trafficking model, Randolph *et al.* showed that monocytes cultured with endothelium, especially after phagocytosing particles, became DCs and then migrated ablumenal-to-luminally. In this same model, it was shown that monocytes that stayed in the sub endothelial network differentiated into MΦs (Randolph et al., 1998). In addition, in vitro experiments performed by Palucka *et al.* demonstrated that MΦs and DCs are capable of interconverting until very late in the differentiation pathway. The authors suggest that the cytokine environment may provide the signal that designates the cells final fate (Palucka et al., 1998).

Is it possible that such a conversion into DCs underlies the ability of MΦs to stimulate immune responses in our experiments? First, it should be noted that even if this were to occur, our data would still indicate that in tissues, MΦs (i.e. before converting to

DCs) are another APC that function in tissues as sentinel cells for immune surveillance. Moreover, we find that stable MΦ clones are also able to directly prime naive T cells.

Our studies have used peptide-pulsed MΦs to demonstrate the potential of these APCs to initiate immune responses. Is it likely that MΦs can stimulate primary T cell responses to other kinds of antigen? Several lines of evidence argue that the answer to this question is yes. It is well established that MΦs acquire antigens from pathogens *in vivo* and present them to T cells. This is basis for host defense against many intracellular pathogens and type IV hypersensitivity responses. Moreover, MΦs isolated from animals injected with proteins can be shown to have processed and presented antigen on both MHC class I and II molecules (Grant and Rock, 1992). Therefore, MΦs clearly acquire antigens *in vivo* and generate peptide MHC complexes. In addition, MΦs in peripheral tissues that have acquired foreign material migrate to regional lymph nodes (Kotani et al., 1979; Shi and Rock, 2002) and we confirm this ability of antigen-bearing MΦs to migrate into secondary lymphoid organs. MΦs that reside in the lymph nodes and spleen also perform a filtering function and capture antigen present in blood or lymph (Maino and Joris, 2004). Such MΦs are present in the T cell zones of the secondary lymphoid organs (Maino and Joris, 2004). Therefore, MΦs do acquire antigens from peripheral tissues, process and present them as peptide-MHC complexes in T cell regions of secondary lymphoid organs. Our data demonstrate that presentation of such peptide-MHC complexes by MΦs will prime T cell responses.

Are MΦs and DCs the only cells that can initiate T cell responses? Kundig *et al.* have shown that fibroblasts transfected with viral proteins directly induced the generation

of viral specific CTL when injected directly into the spleen (Kundig et al., 1995). This observation suggests that a key factor making an APC a "professional" APC is its ability to home to the secondary lymphoid organ allowing it to encounter many T cells in the perfect environment. In addition, a new cell type termed fibrocyte, a blood-borne fibroblast like cell, has recently been demonstrated to be able to prime naive T cells. These cells were found to express collagen receptors as well as express many of the same markers as professional APCs such as MHC II and costimulatory molecules. It has also been shown that fibrocytes can migrate to the lymph node and present antigen with an efficiency approaching that of DCs (Chesney et al., 1997). These experiments suggest that any antigen-bearing cell that migrates to lymph nodes can initiate T cell responses. However, since many experiments have demonstrated that bone marrow-derived APCs are necessary for the initiation of immune responses (Huang et al., 1994a; Huang et al., 1994b; Iwasaki et al., 1997; Sigal et al., 1999; Sigal and Rock, 2000), fibroblasts are unlikely to play a role in the priming of T cell responses in physiological situations. In addition, our experiments using T cells as APCs demonstrate that the ability of an antigen-bearing cell to migrate into the lymph nodes is not sufficient to initiate responses. These T cell APCs, when pulsed with peptide, present as many peptide-MHC complexes as MΦs and upon injection *in vivo* migrate to paracortical (T cell) region of the lymph node; however, they fail to directly prime immune responses. It is possible that B cells can prime naive T cell responses *in vivo*, although this has been controversial. The ability of B cells to prime naive T cells has not been tested in our system. However, it is known that B cells are poorly phagocytic and usually don't cross-present antigens (Rock

et al., 1993); therefore they are unlikely to be important for initiating response to many microbial pathogens and cell-associated antigens.

Summary for T Cell Priming Studies

Because bone marrow APCs play an essential role in detecting infection and initiation of responses, these observations raise many important questions. In what setting do MΦs initiate immune responses? Do T cells stimulated by MΦs exhibit different specificities than those primed by DCs? Since these two APCs may make different mediators and thereby influence responses in different ways, the initiating APC may play a role in determining whether protective immunity is generated. Are the T cells primed by MΦs functionally different from the T cells primed by DCs? It is possible that different APC will process antigen differently and prime different repertoires of T cells. These differences could affect the affinities and avidities of the TCR of T cells primed by the different APC subsets. This would be an important distinction since, e.g., there is evidence that higher affinity T cells are more likely to clonally exhaust. These lower affinity/avidity T cells would play an important role in disease states were there is a high load of antigen like hepatitis or HIV. It will also be of interest to examine the interactions and synergies between different APCs.

The Role Of Fas And Perforin In APC Disappearance

When we began these experiments very little was known about the fate of the antigen-presenting cell after presentation. A couple of studies had observed the disappearance of the APC in animals that had received transgenic T cells specific for the peptide used to pulse the transferred APC. As discussed above, Inguilli *et al.* had

demonstrated the absence of peptide-pulsed DCs in the draining lymph nodes of animals that had received $CD4^+$ transgenic T cells specific for that peptide, suggesting that antigen presentation is a terminal event for DCs (Ingulli et al., 1997). Another study, designed to examine why DC immunization could not cause tumor rejection suggested that this phenomenon also applied to $CD8^+$ T cells. Using a FACS method to quantitate CFSE labeled adoptively transferred SIINFEKL pulsed DCs in the lymph nodes of OT-I mice, Hermans *et al.* also discovered that the T cells eliminate the epitope specific antigen presenting cells, therefore limiting the stimulatory ability of the peptide-pulsed DCs (Hermans et al., 2000). The mechanism of disappearance was not elucidated, but since these were $CD8^+$ T cells the role for both Fas-FasL and perforin was possible. Another bias in this study was the use of the OT-I transgenic animal as a host. In these animals <90 % of the T cells are specific for the antigen in question, so elimination of the DC may not be a physiologically relevant event. Both of these studies speculated on the possible mechanisms involved, but this question was not investigated (Hermans et al., 2000; Ingulli et al., 1997).

A study done by Loyer *et al.* investigated the mechanism of DC disappearance by looking at the role of Fas and Perforin. In their study they used wild type mice as hosts in order to have a normal T cell repertoire. They transferred transgenic T cells specific for the male HY antigen and peritoneal MΦs as APCs into the host animals. Using this protein, they were able to examine the fate of the APC carrying a dominant epitope versus a nondominant epitope. In this system, the authors found that APCs presenting immunodominant epitopes did indeed disappear, very shortly after entering the lymph

node, but only when presenting to MHC I restricted T cells. In contrast, APCs presenting non-dominant epitopes were not eliminated. These authors went on to show that although Fas-FasL interactions played no role in the observed APC elimination, perforin played a partial role. These results suggest that the epitope presented may dictate the fate of the presenting cell and that when immunodominant epitopes are used it may not be possible to extrapolate the results to other epitopes (Loyer et al., 1999). In contrast to these findings, Kawamura *et al.* showed that Langerhan cells were eliminated within 48 hours from the lymph node when they were used as APCs and that this elimination was delayed, but not absent in Fas or FasL deficient mice. These studies additionally demonstrated that the Langerhan express Fas in the draining lymph node and that activation of the cells with IFN led to the further upregulation of Fas expression (Kawamura et al., 1999; Kawamura et al., 2000).

In 2001, a study performed by Ludewig *et al.*, the role of Fas and perforin in the observed APC disappearance was investigated in a LCMV model. This group took advantage of the H8 mouse, which expresses the immunodominant epitope from LCMV's gp33 constitutively. By adoptively transferring these cells into DC primed primed animals they showed they were unable to boost the gp33 specific responses. This group went on to show that this inability to boost the immune responses by repeated immunization with peptide-pulsed DCs was because the APC were eliminated. Additional experiments demonstrated that although DC killing *in vitro* was clearly dependent on perforin, neither perforin nor fas played a role *in vivo* (Ludewig et al., 2001).

Several conclusions can be made from the data in the literature and our data. Our study demonstrates that if Fas-FasL interactions play a role, it is a small one and is only evident at lower antigen concentrations. In addition, our experiments as well as the observations made by Ludewig *et al.* argue against a major role for perforin in this observed APC elimination (Ludewig et al., 2001). Our data also demonstrates that when both Fas and Perforin are not available there is a reduction in the $CD8^+$ T cells' ability to eliminate the APCs at lower antigen concentrations. It is interesting to note that ~50% of the pulsed APCs are still eliminated in the absence of both effector molecules. Therefore, it is clear that multiple redundant mechanisms are involved in the antigen specific elimination of DCs.

These disagreements about the contributions of Fas and perforin to the observed DC disappearance between the various studies may be due to multiple factors. First and foremost, maybe $CD4^+$ T cells and $CD8^+$ T cells utilize different mechanisms to eliminate the peptide pulsed APCs. Another possibility is that the nature of the antigen and or whether or not it is a dominant epitope dictates the fate of the presenting APC as suggested by Loyer *et al.* (Loyer et al., 1999). It is possible that in these different studies, different APC subsets are presenting and that these subsets differ in how they are eliminated. And finally, it is still formally possible that the elimination of the presenting APC is not mediated by the activated antigen specific T cells, but is an intrinsic activity of the APC when it is signaled that its job is done.

The issue of how the longevity of DCs affects immune responses is an interesting one for many reasons. The half-life of DCs may determine how long T cells are

stimulated and thereby influence responses. It is possible that by extending the life of the APC, the number of T cells stimulated could be greatly increased. This increase in stimulated T cells could result in an increase in the number of effector T cells and therefore memory cells. On the other hand, it is also possible that the potential increase in activated T cells may result in an increase in T cells undergoing activation induced cell death or in clonal exhaustion. These questions still need to be investigated and the adoptive transfer model could prove useful in these experiments. The ability to increase the number of activated T cells while decreasing the magnitude of the contraction stage would allow for the formation of a larger memory pool. The ability to manipulate APCs would give immunologists another possible place to make vaccines more effective. How to increase effector cell numbers and increase effecter memory has become the "holy grail" of vaccine development.

Other interesting questions still remain. If the DC is indeed killed by the activated antigen specific T cells, what molecules play a role? Are the molecular "players" different for $CD4^+$ verses $CD8^+$ T cells? Other TNF receptor family members such as TRAIL are potential candidates that have yet to be tested. If death is intrinsic to the DCs, a role for molecules involved in the regulation of the apoptosis such as Bcl-2 family members will need to be investigated. Another important question is are all APCs eliminated after they present antigen? It is possible that MΦs and B cells may or may not be subject to the same fate as DCs. It will also be interesting to investigate whether T cells use different effector molecules to eliminate different APCs.

CHAPTER XIII: REFERENCES

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