THE EFFECTS OF IMATINIB MESYLATE ON ANTIGEN-SPECIFIC CD8$^+$ T CELL RESPONSES

APPROVED BY SUPERVISORY COMMITTEE

James Forman, D.M.D., Ph.D.

Richard Scheuermann, Ph.D.

Michael Bennet, M.D.

John Schatzle, Ph.D.

Robert Ilaria, Jr., M.D.

DEDICATION

To my husband, Christoph and

my parents, Faranik and Ali

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THE EFFECTS OF IMATINIB MESYLATE ON ANTIGEN-SPECIFIC $CD8^+$ T CELL RESPONSES

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PARISA SINAI

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Parisa Sinai, Ph.D.

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Supervising Professor: James Forman, D.M.D., Ph.D.

Imatinib mesylate (IM) is a chemical compound designed to inhibit the constitutive tyrosine kinase activity of the Bcr-Abl oncogene in Chronic Myelogenous Leukemia (CML). While IM is very potent in treating CML, little is understood concerning the effects of IM on the immune response. In this study, I have examined the influence of IM on antigen specific CD8 T cells. Mature OT-1 TCR transgenic T cells were transferred into B6.Thy1.1 recipients. This transfer was followed by an infection with *Listeria monocytogenes* (LM) expressing the cognate epitope OVA $_{257\text{-}264}$ (LM-OVA) to assess whether IM affects the specific CD8 T cell response generated to this intracellular pathogen. *In vitro* studies

revealed that IM had no effect on proliferation, apoptosis, or IFN-γ secretion of naïve or memory OT-1 T cells at doses of ≤5μM, although at higher doses inhibition was observed.

Adoptive transfer and *in vivo* infection studies demonstrated that in the presence of IM the primary response of OT-1 T cells *in vivo* to LM-OVA infection was unaltered as measured by OT-1 T cell percentages in spleen and blood, their expression of IFN-γ and their proliferation. However, IM influenced the primary OT-1 response by decreasing the expression of the IL-7Rα memory marker on OT-1 cells.

IM treatment for >28 days resulted in a decreased percentage of OT-1 memory T cells before recall in blood. Furthermore, the response of memory OT-1 cells after LM-OVA re-challenge was diminished as measured by OT-1 T cell percentages in blood. In addition, IM treatment reduced the expression of IL-7Rα, a receptor required for memory cell survival, on effector and memory OT-1 cells. The function of memory OT-1 cells as measured by IFN-γ expression was unaltered as was their proliferation as measured by incorporation of Brdu. Infection assays revealed that clearance of LM-OVA by memory mice is not altered by IM. In addition, the *in vivo* proliferation of memory OT-1 cells was not altered by IM. While IM did not alter the percentage of CD4 T cells in spleen and blood, IFN-γ expression by CD4 T cells after recall was decreased. Together, these data demonstrate that IM reduces the number of OT-1 cells following a secondary challenge to an LM-OVA infection, and this may be a result of decreased IL-7Rα expression on effector and memory OT-1 cells.

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LIST OF DEFINITIONS

The following list does not include standard abbreviations published in the first issue of each volume of *The Journal of Immunology*.

IM	Imatinib Mesylate
CML	Chronic Myelogenous Leukemia
LM	*Listeria monocytogenes*
LM-OVA	LM engineered to express ovalbumin
DLI	Donor lymphocyte infusions
BMT	Bone marrow transplant
BMT	CML murine bone marrow transduction/transplantation model
SCT	Stem cell transplantation
GVHD	Graft versus host disease
GVL	Graft versus leukemia
7-AAD	7-amino-actinomycin D
AICD	activation-induced cell death
BHI	brain-hear infusion
FADD	Fas-associated-via-Death Domain
IL-7Rα	interleukin 7 receptor α chain
IL-7Rα^{hi}	the cells expressing high levels of interleukin 7 receptor α chain
LCMV	lymphocytic choriomeningitis virus

LLO	listeriolysin O
MFI	Mean fluorescence intensity
VSV	vesicular stomatitis virus
VV	vaccinia virus
OT-I.PL	OT-1 cells on B6.Thy1.1 background
OT-II.PL	OT-II cells on B6.Thy1.1 background
Ph^+	Philadelphia chromosome positive
GIST	Gastrointestinal stromal tumors

CHAPTER ONE

Introduction

Imatinib mesylate (IM) is a tyrosine kinase inhibitor that has become the standard of care for the treatment of patients with chronic myelogenous leukemia (CML). IM inhibits the constitutive tyrosine kinase activity of BCR-ABL, the hallmark oncogene associated with the initiation of CML. With its substantial potency, IM has improved the survival rates for chronic phase CML. It has become part of therapies for other cancers such as gastrointestinal tumors, myeloproliferative and eosinophilic disorders and mast cell disease. In the sections that follow, I will first discuss CD8 T cells, with a focus on memory CD8 T cells. Next, I will discuss *Listeria monocytogenes* (LM) infection and the immune responses to LM. To gain a better understanding of the history of IM, I will then discuss the CML disease. This section includes current therapies for CML, murine models and immunity to CML. A discussion on IM and immune responses to IM will follow next.

I. CD8 T cells

Programming of Naïve CD8 T cells

Naïve CD8 T cells which have undergone positive and negative selection, emerge from the thymus and home to lymphoid organs. Professional APC, such as DC, trap the

antigen in the periphery, undergo a maturation process and move to secondary lymphoid organs(von Andrian and Mackay, 2000). Mature APC, with increased expression of costimulatory molecules and MHC, are efficient stimulators of T cells. In the lymphoid organs, naïve T cells encounter foreign antigen in context of MHC presented by APC, and in the presence of costimulation, proliferate and differentiate into effector cells. The initial 24 hours of antigen exposure is sufficient to commit naïve CD8 T cells to differentiate into effector cells with effector functions of producing IFN-γ, TNF-α, IL-2 and other inflammatory cytokines (Kaech et al., 2002b; Gett and Hodgkin, 2000). This period leading to T cell activation and the killing of infected cells is termed the primary response.

Once naïve T cells receive appropriate TCR and costimulatory signals, they become committed to clonal expansion and differentiation into effector cells. This period is termed the expansion phase (Figure 1). The expansion phase is further followed by the contraction and the memory phases. The differentiation, contraction and memory events are programmed early on. Studies focusing on the duration of antigenic stimulation remove T cells from antigen at different time points. These studies show CD8 T cells stimulated for 24 hours can commit to at least 7-10 division cycles (Kaech and Ahmed, 2001; Mercado et al., 2000). In these experiments, only the parental naïve CD8 T cells were exposed to antigen, and this initial encounter was sufficient to sustain proliferation in the daughter cells in absence of further antigenic stimulation. This indicates that once CD8 T cells reach a certain threshold of activation, a programmed proliferative response follows. It is also during this activation period that the onset of contraction is

programmed (Badovinac et al., 2002). Moreover, studies using adoptive transfer of effector T cells show that CD8 T cells stimulated for only 24 hours, not only proliferate and differentiate into effector cells without further antigenic stimulation, but continue to develop into long-lived, protective memory CD8 T cells (Mercado et al., 2000; Kaech and Ahmed, 2001).

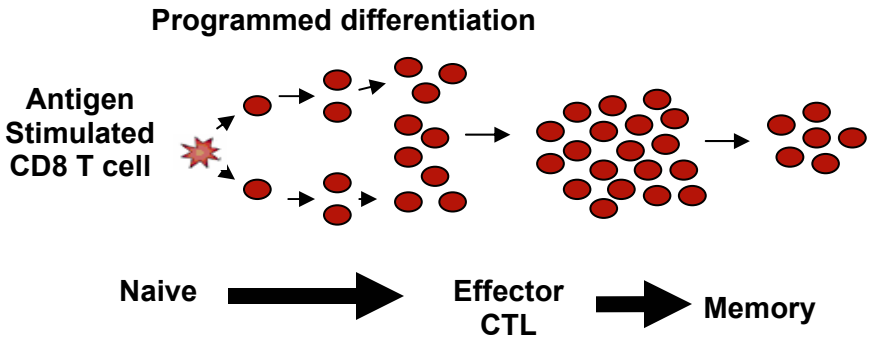

Figure 1. $CD8^+$ T cells are programmed early during development to divide and to differentiate into effector and functional memory $CD8^+$ T cells. An antigenic stimulus triggers a developmental program, so that the $CD8^+$ T cells become committed to proliferation and differentiation. Further antigenic stimulation of the daughter cells may increase the number of times the activated $CD8^+$ T cells divide, but it is not essential to complete this developmental program.

During the first 24 hours of stimulation, CD8 T cells prepare for clonal expansion but no cell division is observed. Soon after, CD8 T cells divide at the rapid rate of 6-8 hrs/ division and the number of antigen-specific CD8 T cells increase rapidly, expanding > 1000 fold (Busch et al., 1998; Hou et al., 1994). During an acute infection with LCMV, CD8 T cells divide about 15-20 times, demonstrating the high proliferative potential of CD8 T cells.

Naïve cells have cell surface protein markers that change, either upregulate or downregulate, upon activation, and change again upon differentiation into memory cells. Naïve CD8 T cells are $CD25^-$, $CD44^-$, $CD69^-$, $CD62L^+$, $CD122^-$, $Ly6C^{low}$ and $CD94^-$. When CD8 T cells become activated these protein markers change to $CD25^+$, $CD44^+$,

$CD69^{+}$, $CD62L^{-}$, $CD122^{+}$, $Ly6C^{-}$ and $CD94^{veryhi}$. Further, when memory T cells develop, they become $CD25^{-}$, $CD44^{+}$, $CD69^{-}$, $CD62L^{hi}$, $CD122^{+}$, $Ly6C^{hi}$ and $CD94^{+}$. A subset of memory cells also bear high levels of CCR7 (the lymph node homing receptor) and CD27 (costimulatory molecule). CD25 is the α chain of the IL-2R that is expressed upon activation(Lipkowitz et al., 1984). CD44 aids in leukocyte extravasation and is upregulated upon activation on both activated and memory T cells (Haynes et al., 1989). CD69 is upregulated on activated T cells and promotes lymphocyte retention in lymphoid organs (Shiow et al., 2006). Meanwhile, the expression of CD62L (L-selectin) molecule which guides naïve and memory T cells to lymph nodes and Peyer's patches, is downregulated on effector T cells, but upregulated again on a subset of memory T cells (Reimann et al., 1991; Reimann et al., 1991). Other markers used to identify effector and memory T cells are CD43 which regulates T cell trafficking, and Ly6C whose function is not known (von Andrian and Mackay, 2000; Onami et al., 2002).

As naïve cells differentiate into effector cells, their cell adhesion and chemokine receptor expression patterns change to allow them to migrate to inflamed tissues. These migration patterns change again on memory cells to allow extravasation into non-lymphoid tissues and mucosal sites (Moser and Loetscher, 2001). Chemokine receptors such as CCR2, CCR5, CCR4, CCR8, CXCR2 and CXCR4 all assist migration of effector and memory T cells to the site of inflammation (Woodland and Dutton, 2003). After about 24 hours of stimulation, the levels of lymph node homing receptors CCR7 and CD62L remain high on activated T cells. As the activated cells become effector T cells, they show a decreased expression of CCR7, and CD62L, and therefore, they have a

reduced potential to home to lymph nodes (Northrop and Shen, 2004). Effector cells have a high expression of chemokine receptors CCR5 and CCR2, and therefore can migrate to inflamed tissues. Cytokines, such as IL-2 and IL-15, modulate T-cell migration patterns by decreasing or increasing the level of expression of CCR7 and CD62L, respectively (Schluns and Lefrancois, 2003). In addition, CD54 (ICAM-1) and CD11a (LFA-1) adhesion molecules are upregulated to facilitate the retention of T cells in the inflamed areas (Shimizu et al., 1991).

One of the molecules that is upregulated on activated and memory cells is CD94. Depending on the NKG2 molecule that CD94 dimerizes with, either an inhibitory or an activating receptor is formed. CD94/NKG2 receptors belong to a group of C-type lectins, and are expressed on ~50% of a mature spleen NK cells and a small subset of CD8 T cells (Vance et al., 1998; Takei et al., 2001). These receptors bind to Qa1, nonclassical MHC I molecule in mice, and its homologue, HLA-E in humans (Takei et al., 2001; Brooks et al., 1999). In mice, most CD94/NKG2 receptors on CD8 T cells are the inhibitory CD94/NKG2A heterodimers. Although very few CD8 T cells from a naïve mouse express CD94/NKG2 receptors, 50% of CD8 T cells undergoing a secondary response against LM are $CD94^{hi}$ and bind the Qa-1b/Qdm tetramer (Gunturi et al., 2003). Expression of CD94/NKG2 is correlated with a lower level of apoptosis and may play a role in maintenance of CD8 T and NK cells (Gunturi et al., 2003). TGFβ increases the expression of CD94/NKG2A on CD8 T cells when there is a concurrent stimulation through the TCR (Gunturi et al., 2005).

Contraction and Memory Phase

As naïve cells develop into activated T cells and expand, they gain the ability to clear the pathogen. The contraction phase follows pathogen clearance (Masopust et al., 2004; Badovinac et al., 2004). During contraction, over 90% of the effector T cells die by apoptosis, and the surviving T cells enter the memory phase. During the memory phase, the number of memory T cells stabilizes and is maintained indefinitely. Properties that distinguish memory T cells from their naïve counterparts and contribute to long-lived immunological protection from re-infection are (1) faster response, (2) faster cytokine secretion, (3) increased longevity, (4) amplified abundance (increase in number of Ag specific cells), and (5) disseminated anatomical location.

Multiple mechanisms are involved in contraction, or the death phase, of effector CD8 T cells. A decline in cytokines that support clonal expansion and T-cell survival (such as type 1 IFN, IL-2, IL-4, IL-7 and IL-15) can lead to the contraction phase (Blattman et al., 2003). Murine models deficient in Fas (CD95), TNFR1 or both show minimal effects on effector-cell death and memory-cell set-points. This suggests pathways other than Fas or TNF can mediate death of effector cells (Zimmermann et al., 1996; Reich et al., 2000). CD40-CD40L interactions regulate memory T-cell set-points by interfering with the contraction phase. In CD40L $(CD154)^{-/-}$ mice, the death of effector CD8 T cells is enhanced and approximately tenfold fewer memory T cells are formed after LCMV infection (Borrow et al., 1996; Whitmire and Ahmed, 2000).

Studies suggest the more target cells a CD8 T cell kills, the less likely it is to develop into a memory cell (Opferman et al., 2001). Both IFN-γ and perforin are important not

only for controlling an infection, but for regulating effector-cell numbers after infection (Badovinac et al., 2000). Perforin$^{-/-}$ and IFN-$\gamma^{-/-}$ mice have increased numbers of CD8 T cells in the contraction phase, indicating that these effector molecules are involved in T cell death (Badovinac and Harty, 2000; Matloubian et al., 1999). Anti-apoptotic and pro-apoptotic molecules also play a role in contraction. For example, the expression of Bcl-2, an anti-apoptotic protein highly expressed in naïve T cells, decreases in effector cells as they undergo contraction (Grayson et al., 2000).

The contraction phase leaves a population of CD8$^+$ T memory cells (T_M) that stably persist after clearance of the infection. Upon a second encounter with the same pathogen, CD8$^+$ memory cells divide rapidly and immediately exert effector function. In addition, they give rise to a large population of secondary effectors. Memory T-cell populations are not generated from a subset of effector cells that 'divide-out' during the contraction phase, but rather, are formed directly from the effector cells themselves (Jacob and Baltimore, 1999). The use of CRE/LOXP system in transgenic mice to mark the virus specific effector T cells showed that the marked cells were maintained in the memory pool (Jacob and Baltimore, 1999). Another approach using adoptive transfer of effector T cells, also showed that memory T cells arise directly from the effector cell population (Opferman et al., 1999).

CD8$^+$ T cell memory cells are subdivided into two populations: (1) effector memory T cells (T_{EM}) and central memory cells (T_{CM}). T_{EM} express low levels of CD62L, CCR7 and CD27, whereas T_{CM} express high levels of CD62L, CCR7 and CD27. T_{EM} are primarily found in peripheral tissues, while T_{CM} are found in secondary lymphatic organs.

T_{EM} are capable of producing effector cytokines immediately upon TCR engagement, while T_{CM} have greater proliferative potential upon re-stimulation (Northrop and Shen, 2004).

Results regarding the exact role of T_{EM} and T_{CM} are still in debate. $CD8^+$ memory (T_M) cells isolated from nonlymphoid tissues exhibit higher levels of direct cytotoxicity compared to those isolated from lymphoid tissues (Masopust et al., 2001). This is consistent with them being T_{EM}. Also protective immunity induced by live vaccines correlates with the generation of T_{EM}, whereas heat-killed vaccines induce CD8 T_{CM} and fail to generate either T_{EM} or protective immunity (Lauvau et al., 2001). These results imply that T_{EM} are true memory cells. However, the transfer of sorted T_{EM} ($CD62L^{lo}$) and T_{CM} ($CD62L^{hi}$) cells into separate hosts demonstrates the conversion of T_{EM} into T_{CM}. Transferred T_{EM} slowly acquired the phenotype of T_{CM}, whereas, none of the T_{CM} ever acquired the phenotype of T_{EM}. In addition, only T_{CM} were capable of self-renewal by a low level proliferation (Wherry et al., 2003). T_{CM} were also more efficient in mediating protective immunity (Wherry et al., 2003). This data suggest that T_{CM} are true memory cells and serve as a reservoir for generating large pool of secondary effectors for re-enforcement upon re-infection. More evidence for this model comes from studies examining functional changes, gene expression profiles, and expression of IL-7Rα on CD8 T_M cells (Kaech et al., 2003; Kaech et al., 2002a). T_{EM} and T_{CM} are not separate lineages, and they are a continuum of linear differentiation from T_{EM} into T_{CM} (Wherry et al., 2003).

CD8 T Cell Effector Mechanisms in Pathogen Infections

CD8 T cells play an important role in tumor control and in immunity against pathogen infection. In order to better understand the mechanisms of CD8 T cell immunity in pathogenic infections, several murine models of intracellular bacterial pathogens have been used. These include *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis*. The viral pathogens used to study CD8 T cell immunity include, in part, vaccinia virus (vv), vesicular stomatitis virus (VSV), influenza virus and lymphocytic choriomeningitis virus (LCMV). Studies in these murine models of pathogen infections emphasize the role of CD8 T cells and their effector mechanisms in control of infections.

An activated CD8 CTL kills the target cell bearing antigenic peptide / MHC through two major effector mechanisms, cytokine secretion and lysis of the infected cell. Cytokine secretion refers to secretion of IFN-γ and TNF-α. Studies analyzing pathogen specific $CD8^+$ T cells have shown that secretion of IFN-γ is important in CD8 T cell function (Smith et al., 1991; Subauste and Remington, 1991). IFN-γ is a potent cytokine that activates host defense mechanisms, including activation of macrophages, stimulation of phagocytosis, upregulation of MHC I and MHC II molecules and induction of the immunoproteosome (Bancroft et al., 1987). In addition, IFN-γ promotes differentiation of CD4 T cell into T_{h1} cells, stimulates CD8 T cell cytotoxicity, and induces B cell class switching to IgG_{2a} (Boehm et al., 1997).

TNF-α stimulates phagocytosis by macrophages and induces apoptosis in the target cell (Portnoy et al., 1989; Ashkenazi and Dixit, 1998). It upregulates adhesion molecules

on endothelial cells, leading to recruitment of phagocytes to the site of infection (Henninger et al., 1997). TNF-α production by $CD8^+$ T cells has also been shown to be potent cytokine against LM (White and Harty, 1998).

Lysis of infected target cell occurs by two different pathways, granule exocytosis and Fas-ligand engagement. Granule exocytosis, occurs very rapidly after TCR engagement. Pre-formed granules containing perforin and granzymes migrate to the cell surface, fuse with the cell membrane and release their contents into the tight junction between the two cells (Kagi et al., 1996). Perforin polymerizes on the target cell membrane and creates pores through which water, salts and released granzymes enter the cytosol (Podack et al., 1991; Podack and Kupfer, 1991). Granzymes, namely granzyme B, activate apoptosis of the target cell through activation of caspases, leading to induction of DNA fragmentation and cell death (Darmon et al., 1996; Sarin et al., 1997).

Fas-ligand, expressed on the surface of a CD8 T cell, is a TNF superfamily member (Nagata and Suda, 1995). Fas-ligand crosslinks with the Fas receptor on the surface of infected cells (Los et al., 1995). Fas-ligand engagement initiates target cell death by activation of the Fas-Associated-via-Death-Domain (FADD) pathway and caspase 8 in the target cell (Bodmer et al., 2000). While the granule exocytosis pathway acts on any infected cell, Fas-ligand induced cell death requires Fas expression on the infected cell. The speed of cytotoxic events initiated with FasL versus granzymes is different. While granules containing granzymes and perforin can be released within minutes of TCR stimulation, maximal FasL activity requires about 2 hours of TCR stimulation. This is because expression of new FasL has to be induced inside the cell (Nguyen and Russell,

2001). However, the long half-life of FasL, 2-3 hours, allows the cytotoxic killing to go on in absence of TCR stimulation and can lead to bystander killing (Wang et al., 1996). With the lysis of target cells, the pathogen released into the extracellular environment is phagocytosed by activated macrophages and killed.

The expression of CD8 T cell effector molecules differs in memory and naive cells. One characteristic of the memory CD8 T cell is the fast cytolytic response. Memory T cells, unlike naïve T cells, rapidly express IFN-γ, perforin and granzyme B. This is due to the constitutive expression of genes encoding IFN-γ, perforin and granzyme B in effector and memory CD8 T cells (Grayson et al., 2001). Also, the CD8 effector mechanisms differ in different pathogenic infections. Use of perforin, Fas-ligand, and IFN-γ deficient mice have shown that some effector functions have more of an impact in specific pathogenic infections. IFN-γ secretion is the only requirement for CTL clearance of *T. gondii*, whereas in a *Listeria* infection CTLs from IFN-$\gamma^{-/-}$ mice cleared the infection (Gazzinelli et al., 1991; Harty and Bevan, 1995). Perforin$^{-/-}$ mice have minimal increased susceptibility to primary LM infection, but are severely impaired after a secondary challenge (Kagi et al., 1994a).

Requirements for T cell activation

Once a T cell encounters a foreign antigen / MHC, the engagement of TCR in complex with the CD3 chains transmit signals into the cell. Engagement of co-stimulatory signals, deliver a second signal into the cell, upon which either a T cell reaches its threshold of activation and starts its effector functions, or alternatively T cell

activation is downmodulated. In addition the cytokine environment influences T cell proliferative responses. In this section, factors that influence the T cell activation responses will be discussed.

It is during the early events of T cell activation that the magnitude and nature of T cell responses are determined. Avidity, duration of TCR engagement with peptide / MHC, and abundance of antigen all contribute to control the magnitude of the response. While avid binding of relatively long duration favors activation, a weak and brief encounter does not (Savage et al., 1999; Davis et al., 1998). In addition, engagement of the TCR receptor can induce not only activation and differentiation but also apoptosis of the T cell (Lenardo et al., 1999). Moreover, the events during activation and priming of naïve cells influence the T-cell differentiation program and have consequences on memory T-cell generation and quality. *In vivo* priming is often associated with variables such as the cytokine environment, antigen abundance, TCR affinity, type of antigen presenting cells, and degree of costimulation.

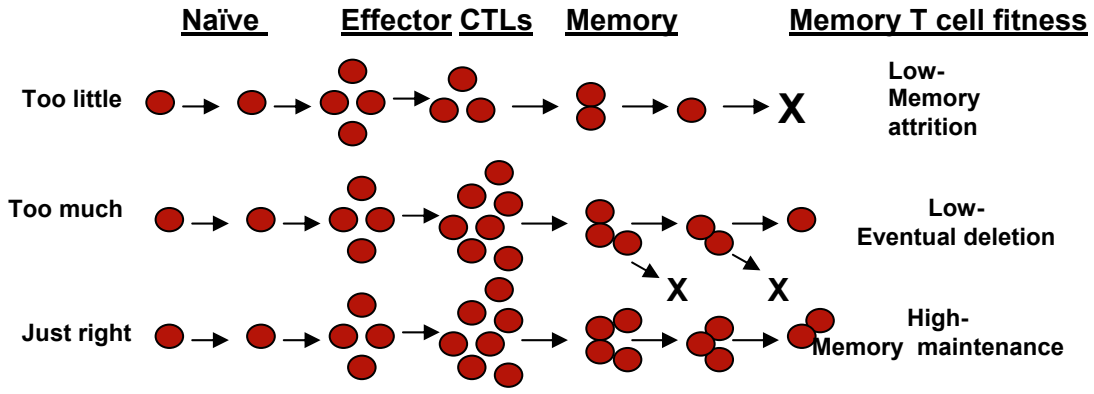

Figure 2. Effector and memory $CD8^+$ T cell development. The first 24 hours after antigen encounter begins a program of expansion and differentiation that continues among daughter cells after removal of antigen. 'Too little' stimulation (insufficient antigen concentration or duration) leads to limited $CD8^+$ T-cell expansion, poor memory development and attrition. 'Too much' stimulation leads to a progressive loss in the ability to secrete cytokines and the eventual deletion

of antigen-specific $CD8^+$ T cells. Optimum memory development happens when conditions are 'just right'; that is, when $CD8^+$ T cells are stimulated by a sufficient concentration of antigen for a sufficient, but not excessive period of time. Several variables, such as the cytokine environment, TCR affinity, type of antigen presenting cells, $CD4^+$ T-cell help and degree of co-stimulation, change the differentiation program and alter the antigen-dependent signaling requirements for the development of a robust response. Several factors encountered during the days following priming, including continued antigen contact, various co-stimulatory molecules, negative regulators, cytokines, chemokines, $CD4^+$ T-cell help and anatomical location may influence the developing response.

Studies in viral pathogenic models indicate that the magnitude of the CD8 response is proportional to epitope abundance. Transfer of equal numbers of TCR-transgenic T cells into mice followed by titration with different doses of stimuli have also shown that the greater the viral antigen load, the larger the number of effector cells produced (Wherry et al., 1999). In the case of chronic LCMV infection in mice, where antigen is present for long periods of time, the rate of proliferation of effector CD8 T cells slows, the cells lose function and some are physically deleted (Zajac et al., 1998). This indicates that the magnitude of CD8 T cell response cannot be maintained indefinitely, and the number and function of effector $CD8^+$ T cells is reduced when antigen levels remain high for extended periods of time (Figure 2).

Another factor involved in control of the magnitude of the response is costimulation. An example is CD40L upregulation induced on activated T cells and its interaction with CD40 on B cells and dendritic cells (Lederman et al., 1992). This interaction causes an upregulation of CD80 and CD86 (Caux et al., 1994; Klaus et al., 1994). Interaction of CD80 and CD86 with their ligand CD28 on T cells causes T cell activation. By contrast, CD80 and CD86 interaction with cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on T cells causes anergy or tolerance. Blockade of either the CD80-CD28 or CD40L-

CD40 pathways induces anergy or tolerance, whereas blockade of CTLA-4 enhances the immune response (Boussiotis et al., 1996; Chambers et al., 2001).

Tolerance and anergy are important mechanisms by which tumors can escape immune recognition. While anergy can be explained as a nonspecific state of inactivation, tolerance could be defined as an antigen-specific state of inactivation. Negative selection is the major mechanism of self tolerance, and it eliminates the autoreactive T cells that can cause autoimmune disease. In the thymus, the T cells are selected for survival because of their ability to bind self peptides with low avidity, and yet they constitute the population of T cells that deals with foreign antigens in the periphery. This can be because a given TCR can cross-react with multiple peptides (Mason, 1998; Garcia et al., 1998). In addition, because foreign antigens are not normally present in the thymus, T cells with potential for high-avidity binding to foreign antigen peptide/MHC evade negative selection and emigrate to the periphery. In the periphery, negative costimulatory signals further contribute to generation of tolerance or anergy.

Besides CD28-CD80 and CD40-CD40L (also known as CD154) interactions, other costimulatory interactions exist. These include 4-1BB-4-1BBL, OX40-OX40L and CD27-CD70 interactions. Costimulation might act early to augment TCR-mediated signaling (such as CD28 or CD40), or later to sustain T cell responses (such as OX40 and 4-1BBL) (Kaech et al., 2002b; Kopf et al., 1999). Signaling through CD40, CD27, 4-1BB and inducible positive costimulatory molecule (ICOS) support continued expansion, survival and memory differentiation of activated T cells (Hendriks et al., 2003; Takahashi et al., 1999). Inducible negative regulators, such as CTLA-4, B and T lymphocyte

attenuator (BTLA) and programmed death 1 (PD-1) attenuate clonal expansion (Greenwald et al., 2002; Watanabe et al., 2003). The majority of T cell co-stimulators belong to either the CD28 or the TNF-receptor family of molecules (Greenwald et al., 2002; Croft, 2003). The ligands of these co-stimulators are expressed on professional APC such as DC, and some ligands are also present on peripheral tissues (Kroczek et al., 2004).

The requirements of costimulation on CD4 and CD8 T cells vary. CD8 T cells seem to be less dependent on costimulation than CD4 cells, but this can also depend on the pathogen. For example, while B7-CD28 costimulatory signals are required for CD8 T cell activation in vesicular stomatitis virus (VSV) and vaccinia virus (VV) infections, they are not required for the priming of CD8 T cells in LCMV infection (Suresh et al., 2001; Mullbacher and Flynn, 1996). CD4 helper T cell responses are completely eliminated in $CD28^{-/-}$, $CD40L^{-/-}$, and $OX40^{-/-}$ mice in viral infection models. While CD8 T cell responses are moderately reduced in $CD28^{-/-}$ and $4\text{-}1BBl^{-/-}$ mice, they are not affected in $CD40L^{-/-}$ and $OX40^{-/-}$ mice. CD40 can also be expressed on CD8 T cells directly (Bourgeois et al., 2002a). This provides for a direct costimulatory effect in the absence of properly activated APCs.

Cytokines also contribute to CD8 T cell expansion, differentiation and survival. Two critical but functionally distinct cytokines are IL-2 and IL-15. IL-2 and IL-15 interact with T cells via cytokine receptor complexes consisting of the common gamma chain (γc), common β chain subunit (CD122), and a unique α chain subunit that confers high affinity cytokine binding (Schluns and Lefrancois, 2003). While CD25 is the IL-2Rα

chain, IL-15 binds with high affinity to IL-15Rα (Dubois et al., 2002; Burkett et al., 2004).

IL-2 is secreted after TCR ligation, and therefore controls clonal expansion by driving mitosis of activated T cells (Cantrell and Smith, 1983). It controls the late clonal-expansion by inducing cell death (D'Souza and Lefrancois, 2003). Furthermore, IL-2 signals during priming are essential for secondary expansion of CD8 T cells (Williams et al., 2006). In addition IL-2 is important for maintenance of tolerance (Malek and Bayer, 2004). By contrast, although IL-15 is involved initially to activate DCs, it plays only a limited role in CD8 T cell expansion *in vivo* (Homann et al., 2001). IL-15, however, is essential for generation and maintenance of memory CD8 T cells (Klonowski and Lefrancois, 2005).

Cytokines important for T cell differentiation and survival are IL-12 and IL-4 along with IL-7 and IL-15. IL-12 promotes development of effector function and survival (Schluns and Lefrancois, 2003). Addition of IL-4 to CD8 T cells activated *in vitro* aids survival and modulates the effector differentiation program (Schluns and Lefrancois, 2003). IL-15 and IL-7 both are survival cytokines. Both IL-15 and IL-7 rescue T cells from cell death at the contraction phase. While IL-15 is important for long term low level proliferation of memory CD8 T cells, IL-7 seems to be more important for promoting survival, rather than growth, of memory T cells (Kang and Der, 2004).

IL-7Rα

IL-7 is a member of γc chain cytokine receptor family that also include IL-2, IL-4, IL-9, IL-15 and IL-21. IL-7R is a heterodimer composed of IL-7Rα and γc chains. The α chain is also a component of the high-affinity receptor complex for thymic stromal lymphopoietin (TSLP). IL-7 is an important cytokine in the generation, maintenance and homeostasis of T cells (Kang and Der, 2004).

IL-7R is involved in transmitting survival signals both in developing and in mature B and T cells (von Freeden-Jeffry et al., 1995). IL-7 pro-survival effects include increasing Bcl-2 expression, repressing Bax expression and activating the PI3-Akt pathway (Kang and Der, 2004; Rathmell et al., 2001). Bcl-2 expression partially rescues hematopoeitic cells in IL-7R$^{-/-}$ mice (Akashi et al., 1997). Also in an IL-7 dependent cell line, cytokine treatment prevents Bax from adopting an active conformation and translocating to the mitochondria (Khaled et al., 1999).

The expression of IL-7R is relatively high in early pro-T and pro-B cells and is then downregulated. It is then re-expressed on mature thymocytes and peripheral T and B cells (Sudo et al., 1993). The IL-7Rα chain is also highly expressed on lymphoid tissue initiating cells (LTICs), essential for generating peripheral secondary lymphoid tissues such as lymph nodes (Yoshida et al., 1999). Experiments that have examined graded doses of IL-7R signaling *in vitro* affirm that IL-7 does not function solely as an on/off switch, but rather acts in a concentration-dependent or threshold regulated manner (Kang et al., 1998). At the pro-T cell stage, partial discrimination of αβ versus γδ T cell lineage biased precursors correlates with IL-7R expression (Kang et al., 1998).

IL-7Rα chain (CD127) has been identified as a marker for memory cells (Kaech et al., 2003). Expression of this marker identifies the precursors of memory cells early in a response (Kaech et al., 2003; Huster et al., 2004). Expression of IL-7Rα is downregulated in most effector antigen-specific CD8 T cells, however, a small subset express high levels of IL-7Rα and anti-apoptotic molecules. The number of IL-7Rα^{hi} cells remain relatively constant during the contraction phase. This small subset identifies the precursors of memory cells. Transfer of IL-7Rα^{hi} cells leads to protective immunity (Kaech et al., 2003; Huster et al., 2004). CD127^{lo} cells resemble T_{EM}, while CD127^{hi} cells progressively acquire the functional and phenotypic characteristics of T_{CM} (Huster et al., 2004).

Molecular links of activation and apoptosis

At the molecular level, the transcription factors T-bet and eomesodermin (Eomes) provide for a link between programming of effector and memory CD8 T cells. T-bet is involved in development of cytotoxic lineages as it plays a crucial role in CD4 T_{h1} differentiation (Szabo et al., 2002). The functions of T-bet, along with those of Eomes, have been linked to regulating genes encoding IFN-γ and cytolytic molecules in CD8 T cells (Pearce et al., 2003; Svensson et al., 2005) (Figure 3). Both transcription factors T-bet and Eomes are necessary for enhanced expression of CD122, the receptor chain that confers cellular responsiveness to IL-15 (Intlekofer et al., 2005).

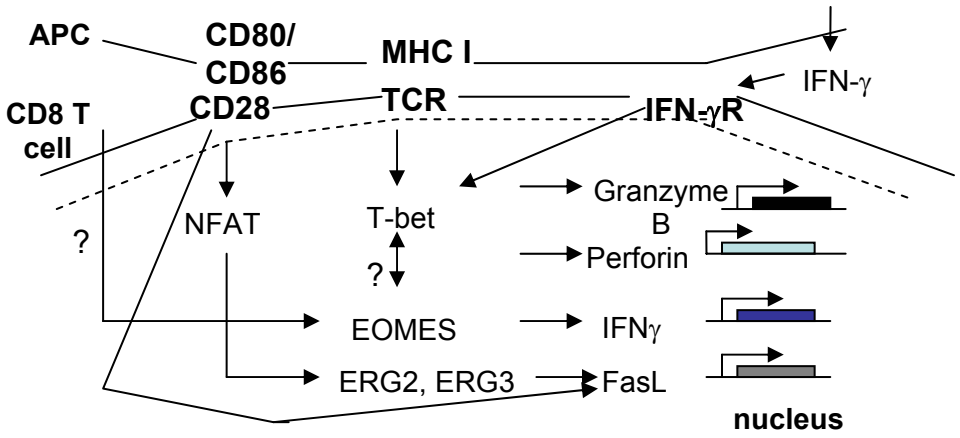

Figure 3. Important transcriptional pathways in CD8$^+$ effector T cells. T-bet expression is induced by signaling through TCR and IFN-γR. Then T-bet induces the expression of the effector molecules IFN-γ, perforin and granzyme B. Eomesodermin (EOMES) also induces the expression of IFN- γ and perforin. There is a CD28-dependent pathway which activates the expression of FAS ligand (FASL) either directly or through the transcription factors NFAT (nuclear factor of activated T cells), EGR2 (early growth response 2) and EGR3.

Unless T cells are stimulated to proliferate, isolated lymphocytes or lymph node cells in culture undergo rapid cell death. To maintain viability, lymphocytes depend on engagement of the TCR, costimulation and/or cytokines receptors. Signaling through these receptors transmits survival signals through separate cascades. The result of these signals is the sequestration of apoptotic factors within mitochondria. Receptors induce survival signals by promoting expression and function of anti-apoptotic Bcl-2 family of proteins or by activating the phophatidylinositol-3 kinase-Akt pathway. Either of these events suppresses the function of pro-apoptotic proteins Bax and Bak. Bax and Bak are required for mitochondrial release of cytochrome c. Disruption of membrane integrity and release of cytochrome c into the cytosol in turn results in activation of caspase-9, thereby inducing the morphologic features of cell death. In addition, survival signals modulate the expression or function of the BH3 regulatory proteins, and sustain the cellular functions necessary for the maintenance of mitochondrial function.

For CD8 T cell lymphocytes, survival receptors include TCR, CD28, IL-2R, IL-4R, IL-7R, IL-9R and IL-15R, IL-6R, c-kit and the ICAM-2 adhesion molecule. Signaling through the IL-2 family of receptors transmits signals through the Jak (1,3)/Stat pathway, causing induction of STAT5, which in turn induces transcription of the pro-survival Bcl-2 gene. The Jak mediated pathway also induces the PI3K-Akt pathway, and Ras and mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK). Antigen receptor and costimulation (CD28) control of survival also is mediated through signaling pathways linked to PI3 kinase and Akt. In addition, receptor signal transduction also supports cell metabolism and glucose uptake. Decline in glucose metabolism results in loss of mitochondrial integrity and release of cytochrome c. Moreover, signaling through many of these receptors shares Akt activity. Activated Akt stimulates glycolysis through its effects on Glut 1 and hexokinase (HK), thereby generating substrates for the mitochondria.

Maintenance of Naïve and Memory CD8 T cells

Naïve CD8 T cells, like naïve CD4 T cells, require both IL-7 and TCR engagement for survival. Naïve CD8 cells are not detectable after transfer into syngeneic recipients that lack MHC I (Goldrath and Bevan, 1999). In addition, mice engineered to control TCR expression show that the absence of the TCR leads to a gradual loss of naïve T cells over 3-4 weeks (Labrecque et al., 2001). These studies confirm the requirement for a continual interaction between TCR and self-antigens. A second signal by IL-7 is also required for the survival of naïve T cells *in vivo* (Schluns et al., 2000). Conventional

costimulatory signals provided by CD28-B7 interactions are not needed for T cell survival (Cho et al., 2000).

$CD8^+$ memory cells are maintained and expanded for long periods by cytokine signals alone, independent of cognate antigen and TCR engagement (Tanchot et al., 1997; Murali-Krishna et al., 1998). Memory CD8 T cells proliferate in MHC $I^{-/-}$ mice and retain their functional properties such as a rapid cytokine response (Tough and Sprent, 1994; Murali-Krishna et al., 1999). IL-$15^{-/-}$ mice can generate antigen-specific effectors and clear viral infections, however, antigen specific memory CD8 T cells do not proliferate in IL-15 deficient mice (Becker et al., 2002). These studies indicate that IL-15 provides essential signals for the survival of CD8 memory cells (Zhang and Ren, 1998; Judge et al., 2002).

Upon transfer into lymphopenic hosts, naïve T lymphocytes undergo substantial division and convert to a memory cell phenotype by homeostatic peripheral expansion (HPE) –referred to as HP memory cells in some studies (Goldrath and Bevan, 1999; Rocha et al., 1989). Both TCR engagement and IL-7 are required for HPE of naïve cells (Ernst et al., 1999). The magnitude of expansion is greatly enhanced during HPE of naïve cells. HPE and conversion to a memory phenotype depends on CD4 help (Hamilton et al., 2006). Studies also show dendritic cells are required to present antigen to cells undergoing HPE (Fry et al., 2004).

CD4 T cell help for CD8 memory T cell generation

The ability of helper cells to augment the activity of cytotoxic T lymphocytes has been studied extensively (Castellino and Germain, 2006; Behrens et al., 2004). Early studies by Cantor and Boyse demonstrated the collaboration between helper cells and cytotoxic T cells bearing different Ly^+ antigens (Ly-1 and Ly-23 T cells) *in vitro* (Cantor and Boyse, 1975). Later, von Boehmer and Haas, demonstrated the need for helper cells in the killer cell response to H-Y antigen (von and Haas, 1979). Using the class I Qa-1 CTL model, Keene and Forman showed the need for linked recognition between helper cells and CD8 cells in priming of Qa1 specific CTL in B6 mice (Keene and Forman, 1982). Later, Cassell and Forman showed for the first time that these helper cells are $CD4^+$. Presentation of antigens for the CTL and CD4 cells on APC enables the CD4 cells to help CTL precursors (Cassell and Forman, 1988).

Since then, studies have suggested a model where CD4 cells are activated by engagement of the TCR with peptide /MHC II on APCs, and interactions of CD40/CD40L costimulatory molecules (Behrens et al., 2004). CD8 T cells are activated by engagement of their TCR with peptide / MHC I on APC, and engagement of B7/CD28 costimulatory molecules. Upon activation, CD4 cells may help the activation of CD8 T cells by secretion of IL-2. IFN-γ secretion by CD4 cells drives CD4 differentiation into the CD4 Th_1 subset, which further helps the development of CD8 cytotoxic T cell responses (Castellino and Germain, 2006). CD4 help to CD8 T cells can both be specific or nonspecific (Castellino and Germain, 2006). Specific help refers to the CD4 response

to the same pathogen that the CD8 response is being generated against. Nonspecific refers to help from a CD4 T cell activated to a different pathogen.

More recent studies show CD4 T helper cells can affect CD8 T cells responses through CD40-CD40L interactions. While CD40-CD40L deficiencies impair CD8 T cell responses, anti-CD40 antibodies substitute for $CD4^+$ T cell help during primary responses (Borrow et al., 1998; Schoenberger et al., 1998). Activated CD4 T cells express CD40L and activate $CD40^+$ APCs. These activated APCs are then able to drive CD8 T cell responses (Lanzavecchia, 1998). CD4 T cell help during CD8 T cell memory responses occurs in response to both helper-dependent and helper-independent antigens (Bourgeois and Tanchot, 2003; Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). Helper dependent antigens are defined as those that can not activate APCs directly and depend on exogenous CD4 T cell help (i.e.: cross-presented antigens). Helper-independent antigens are those that can activate APCs directly such as infectious agents.

CD8 T cells also express CD40 transiently after activation (Bourgeois et al., 2002a). Through the interaction of CD40L on CD4 T cells and CD40 on CD8 T cells, CD4 T cell help for the differentiation of CD8 T cells occurs even when all APCs are CD40 deficient (Bourgeois et al., 2002a; Lu et al., 2000). In addition, CD8 T cells deficient of CD40 do not differentiate into memory cells, demonstrating that helper signals through CD40 molecules on activated CD8 T cells are required for differentiation into memory cells (Bourgeois and Tanchot, 2003; Tanchot and Rocha, 2003). This view, however, is contradicted by studies in the LM and LCMV models that show CD40 deficient CD8 T

cells are fully functional (Sun and Bevan, 2004). Other studies indicate that in the absence of CD40 on CD8 T cells, inflammatory stimuli may substitute for CD40 stimulation (Lee et al., 2003).

The role of CD4 T cells for the development of CD8 T cells has been studied in many different infectious models including: (1) the response of monoclonal $CD8^+$ T cells specific for HY male antigen, (2) the response of CD8 T cells to cross-presented antigens and to LCMV infection, (3) the response of CD8 T cells to VSV infection expressing glycoprotein GP-33 from LCMV, and (4) the response of CD8 T cells to *Listeria monocytogenes* expressing recombinant ovalbumin (Bourgeois et al., 2002a; Bourgeois et al., 2002b; Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). These studies show that CD4 T cells are not required for the development of efficient primary CD8 T cell responses against infectious agents. Division, IFN-γ secretion and CTL activity during the primary response were induced in absence of CD4 T cell help. Specifically, in the LM infection model, the effect of CD4 T cell help on primary CD8 T cell expansion was not detected (Shedlock et al., 2003; Sun and Bevan, 2003).

However, absence of CD4 T cell help produces poor responses of CD8 memory cells. The CD8 cells primed in the absence of CD4 T cell help proliferate poorly upon a secondary infection, and secrete low levels of cytokines (Sun and Bevan, 2003). Long-term maintenance and protective immunity is gradually lost (Sun and Bevan, 2003; Janssen et al., 2003; Shedlock and Shen, 2003). Transfer of CD8 T cells into MHC class II KO, CD4 KO mice, or mice treated with depleting CD4 antibodies, all show that poorer secondary CD8 T cell responses are generated.

In the LM model, however, it is still unsettled as to when during the CD8 T cell response CD4 T cell help is necessary. Some reports indicate that CD4 T cells are crucial only during the initial priming of naïve CD8 T cell differentiation into functional memory cells (Sun and Bevan, 2003; Shedlock et al., 2003). However, other studies show that CD4 T cell help for the CD8 T cell response is essential and plays a role after the primary CD8 T cell response(Sun et al., 2004). Adoptive transfer of effector or memory CD8 T cells into CD4 T cell deficient mice shows that CD4 T cells are required after, not during, the early programming phase (Sun et al., 2004).

Studies suggest that cooperation between CD8 and CD4 T cells involves recognition by antigens co-presented by the same APC (Keene and Forman, 1982; Bourgeois et al., 2002a). But because $CD4^+$ and $CD8^+$ cells specific for a given antigen are rare in the naïve lymphocyte pool, other studies have argued that it is unlikely this interaction is on the same dendritic cell within the lymph nodes (Miller et al., 2004; Mempel et al., 2004). However, antigen-dependent $CD4^+$ T cell interactions with DC in lymph nodes can last several hours, providing a large window of time where a third cell can interact with the $CD4^+$-DC pair (Stoll et al., 2002). Also activated DC produce chemokines that attract CD8 T cells expressing chemokine receptors (Cyster, 2003; Proietto et al., 2004). Subsequently, further work has shown that chemokines, namely CCL3 and CCL4, guide naïve CD8 T cells expressing CCR5 to the sites of $CD4^+$-DC interaction in the lymph nodes. Interference with this guided recruitment markedly reduces the memory CD8 T cell generation (Castellino et al., 2006).

II: *Listeria monocytogenes*

History and pathogenicity

LM is a Gram-positive bacterium that is often used to study the murine immune responses to intracellular infection. The first documented reports of LM date back to United Kingdom in 1926, identifying a Gram-positive bacterium as the cause of lethal disease in a rabbit colony (Pamer, 2004). The second reported outbreak of LM was in South Africa in 1927 as the causative agent of Tiger River Disease, a disease that affects wild gerbils (Pamer, 2004). LM was subsequently discovered to cause human disease. People are most commonly exposed to LM by ingesting contaminated foods, such as unpasteurized dairy products and incompletely cooked meats (Bibb et al., 1990). Immunocompromised individuals and pregnant women are particularly vulnerable to infection.

The natural route of infection with LM is through the gastrointestinal tract. LM infects intestinal epithelial cells by utilizing internalin A, a cell surface protein expressed by the bacterium that binds to E-cadherin, ultimately inducing endocytosis by the host cell (Gaillard et al., 1991). The bacteria then traverses the epithelial cell-layer, enters the blood stream and disseminates to other organs. Once in the spleen and liver, the bacteria induces internalization by splenic and hepatic macrophages. Internalization results from the activation of alternative complement pathway by components of the LM cell wall resulting in deposition of complement protein C3b on the bacterial cell surface (Croize et al., 1993). Macrophages expressing the CR3 receptor phagocytose LM (Drevets et al.,

1992). Inside macrophages, the majority of LM are killed inside the vacuole or phagosome (Portnoy et al., 1989).

However, some LM escapes the phagosome by secreting listeriolysin (LLO), a virulence factor that disintegrates the phagosomal membrane (Bielecki et al., 1990). Once in the cytosol, the bacteria replicates and gains motility. Mobility in the host cytosol is dependent upon the expression of actin-assembly-inducing protein (ActA), a bacterial protein that nucleates actin (Domann et al., 1992; Chico-Calero et al., 2002). By creating actin polymers, the bacteria propels itself through the cytoplasm and into the neighboring cells (Camilli et al., 1993; Cossart et al., 1989). Intercellular mobility contributes to the virulence of LM, while invasion of the cytosol triggers innate inflammatory responses. Immunization of mice with LM strains deficient in LLO does not induce protective immunity, while infection with ActA-deficient bacteria induces innate immune responses and primes protective T-cell responses (Berche et al., 1987; Goossens and Milon, 1992). This confirms that invasion of the cytosol is essential for the development of protective immunity.

LM administered intravenously is taken up from the bloodstream by splenic and hepatic macrophages (Aichele et al., 2003). In the spleen, the marginal-zone LM-infected macrophages migrate to the T cell zones in the white pulp within 24 hours of infection, and co-localize with T cells and LM (Conlan, 1996; Berg et al., 2005). A large number of non-pathogen-specific T cells undergo apoptosis as a result of bacterial transport into the T cell zone (Merrick et al., 1997). In the T-cell zone, LM-infected macrophages and TipDCs (TNF and iNOS producing DCs) meet, creating an

inflammatory response. It is thought that antigens are presented to CD8 T cells in the T cell zone by cross presentation. Furthermore these non-antigen-specific T cells are believed to be an important source of IFN-γ early in LM infection (Berg et al., 2002; Berg et al., 2003). It is possible that the inflammation in the T-cell zone causes the production of IFN-γ along with the subsequent death of T cells.

Innate immunity to LM

The importance of innate immunity in LM infection was shown by using SCID or nude strains of mice (Bancroft et al., 1991). These mice were resistant to infection with LM at early time points but were unable to clear the bacteria over the long term. Neutrophils and macrophages are the principal mediators of killing during the LM innate immune response. Secretion of IL-1 and IL-6 by LM-infected macrophages recruits neutrophils to the site of infection (Dalrymple et al., 1995). In the liver, these neutrophils kill the infected hepatocytes through direct lysis or by inducing apoptosis (Rogers and Unanue, 1993).

Besides neutrophils, macrophages also have to traffic to the site infection. CCL2 is a chemokine produced by LM-infected macrophages. The CCL2 chemokine attracts circulating monocytes expressing chemokine receptor CCR2 to the site of infection. Furthermore, TNFα, IL-12 and IL-18 secreted by LM-infected macrophages recruit and activate NK cells, which in turn produce IFN-γ needed to activate macrophages (Tripp et al., 1993). LM-infected macrophages are also responsible for recruitment of TipDCs, TNF and iNOS-producing DCs (Pamer, 2004). In the spleen, killing during the innate

response involves production of an oxidative burst, nitric oxide (NO) and TNF, produced by uninfected macrophages and TipDCs (Serbina et al., 2003b).

Both IFN-γ and TNF are essential in the primary defense against LM, as IFN-$\gamma^{-/-}$ and $TNF^{-/-}$ mice show increased susceptibility to infection (Rothe et al., 1993; Harty and Bevan, 1995). TNF receptor p55 (TNFRp55), lymphotoxin-β receptor, and TNF-related apoptosis-inducing ligand (TRAIL) each play a role in control of early LM infection (Ehlers et al., 2003; Zheng et al., 2004). Shedding of TNFRp55 controls thresholds of innate immune activation (Ehlers et al., 2003). Mice lacking TNF-related apoptosis-inducing ligand (TRAIL) have enhanced resistance to LM (Zheng et al., 2004). It is also interesting to note that while type I IFNs in viral infections protect the host, they impair the immune response in the case of a bacterial infection. Mice that lack the receptor for type I IFNs are more resistant to LM infection (Carrero et al., 2004; O'Connell et al., 2004). Loss of TNF-producing cells, in addition to apoptosis of T cells can account for decreased bacterial clearance in presence of type I IFN signaling (Pamer, 2004). Furthermore, type I IFNs also decrease the viability of LM-infected macrophages (Stockinger et al., 2002). These data suggest type 1 IFNs are important in LM innate response.

Toll-like receptors also mediate signals in response to an LM infection . Both TLR2 (involved in the recognition of bacterial peptidoglycan, lipoteichoic acid and lipoproteins), and TLR5 (the receptor for bacterial flagellin), are involved in recognition of LM (Seki et al., 2002). MyD88 is the essential downstream signaling component of TLR2 and TLR5, and is therefore essential for the innate immune response against LM

(Seki et al., 2002; Edelson and Unanue, 2002). Macrophage mediated killing of LM does not require MyD88 (Edelson and Unanue, 2002). Instead, MyD88 mediated signaling in TipDC is essential for production of iNOS or TNF during the innate response (Serbina et al., 2003a).

Adaptive immunity to LM

In the adaptive response to an LM infection, αβ T cells are the main contributors to bacterial clearance and to long term protection (MACKANESS, 1962). Adoptive transfer studies of LM specific CD4 or CD8 cells show that CD8 cells play a larger role in providing long-term protective immunity than CD4 cells (Harty et al., 1992; Ladel et al., 1994). While CD4 mediated protective immunity requires IFN-γ production by T cells, CD8 mediated protective immunity can work independently of IFN-γ (Ladel et al., 1994; Harty and Bevan, 1995). CD8 T cells in the LM infection model are divided into two groups: CD8 cells restricted by MHC class Ia, and CD8 cells restricted by MHC class Ib molecule H2-M3 (or the nonclassical). In the sections that follow each of these responses will be discussed briefly.

In the class Ia response, many of the antigenic peptides are generated from LM secreted proteins, many of which contribute to bacterial clearance (Finelli et al., 1999). Two of the known antigenic proteins that induce a substantial CD8 T cell response are LLO (the hemolysin involved in escape from the phagosome) and p60 (a hydrolase involved in bacterial septation) (Villanueva et al., 1995; Bubert et al., 1992). Of the antigenic peptides investigated in LM, three peptides originate from LLO and p60, while

the fourth peptide is from a metalloprotease (Busch et al., 1997; Pamer et al., 1991; Pamer, 1994). While DCs are not targets of an LM infection, it is thought that they prime the CD8 T cells by cross-presentation of antigens derived from infected macrophages (Jung et al., 2002).

During an LM response, priming of naïve T cells is markedly influenced by costimulation with CD28, 4-1BB (or CD137) and ICOS. Both CD28 and 4-1BBL deficient mice display reduced antigen-specific CD8 T cell responses (Shedlock et al., 2003). CD8 T cell responses are also impaired when ICOS is blocked with a soluble ICOS-Ig fusion protein (Mittrucker et al., 2002). CD40-CD40L interactions may have a distinct contribution, although this is still under investigation. Blocking the CD40-CD40L interaction does not prevent priming of CD8 cells, the generation of memory, or long-term protective immunity by CD8 T cells (Sun and Bevan, 2004; Hamilton et al., 2001). In contrast, in absence of CD40 mediated signaling, the frequency of antigen specific CD8 T cells in the mucosa is reduced (Pope et al., 2001). Furthermore, early CD40L-dependent signaling contributes to the generation of a distinct subset of CD8 memory T cells (Huster et al., 2004).

The mechanisms of T cell mediated killing in an LM infection are distinct in different tissues. CD8 T cells that lack perforin and TNF provide protection against the infection in the liver, but not the spleen (White et al., 2000). Furthermore, perforin-deficient CD8 T cells in the spleen provide immunity by a mechanism independent of Fas but require TNF-α (White and Harty, 1998). These imply that perforin and TNF together are more important in bacterial clearance from the spleen. By contrast, in the liver, FAS-mediated

cytolysis is important in bacterial clearance (Jensen et al., 1998). Furthermore, perforin is important in a secondary response to LM. While perforin $^{-/-}$ mice have a slight increased susceptibility to a primary infection, they are unable to clear a secondary infection completely (Kagi et al., 1994b). In addition, in the cellular response to LM, the expression of IFN-γ by LM-specific CD8 T cells is not important for protection (Harty and Bevan, 1995; White et al., 2000).

The effector functions of LM-specific T cells influence their in vivo expansion and survival. In support of this, the magnitude of class Ia response is influenced by both IFN-γ and perforin, as in the absence of these two proteins the specific CD8 T cell response is increased (Badovinac and Harty, 2000; Badovinac et al., 2000). The peak of LM bacterial burden in spleen is 3-4 days after infection, and antigen-specific CD8 T cells are detectable starting on day 4. However, interestingly, *in vivo* optimal priming of CD8 T cells occurs only 1 day after infection (Wong and Pamer, 2003). In addition, infusion of naïve LM-specific CD8 T cells into mice indicate that LM specific CD8 T cell responses contribute to a feedback regulation of antigen presentation, limiting further activation of T cells (Wong and Pamer, 2003).

CD8 T cell programming studies in the LM model indicate that early innate responses influence the contraction phase. However, innate responses occurring after the first 24 hours have only a small impact on the CD8 T cell response (Badovinac et al., 2004; Busch et al., 2000). Programming of the CD8 T cell occurs during the first few days of LM infection (Badovinac and Harty, 2002; Mercado et al., 2000). Furthermore, during the LM response, there is an antigen-independent T cell proliferation, involving secretion

of IL-2 (Wong and Pamer, 2001; Kaech and Ahmed, 2001). In addition, once a threshold quantity of LM-derived antigen has been presented, the magnitude of the T-cell response is unaffected by increased presentation of antigen. Experiments using mice treated by antibiotics to clear the LM infection show that T-cell responses in the LM-model are independent of duration or dosage of antigen exposure *in vivo* (Mercado et al., 2000; Badovinac and Harty, 2002).

Immunization with heat-killed LM (HKLM) induces proliferation of LM-specific CD8 T cells, but does not induce protective immunity (von Koenig et al., 1982). It seems that T cells primed with HKLM undergo division, although attenuated, and do not acquire effector functions (Lauvau et al., 2001). Both CD40-mediated signals and DC expression of costimulatory molecules provide for a positive stimulus induced by live but not heat killed immunization (Rolph and Kaufmann, 2001). Also mice that were concurrently immunized with live LM and HKLM, show that T cell activation in the spleen in compartmentalized (Lauvau et al., 2001).

CD4 regulatory cells have been implicated in control of LM infection. Depletion of $CD25^+$ $CD4^+$ cells enhances the memory CD8 T cell responses (Kursar et al., 2002). CD4 T cells are not required for the development of efficient primary CD8 T cell responses against LM. However, without CD4 T cells, memory CD8 T cells produce a poorer secondary response than do memory CD8 T cells generated with CD4 help (Sun and Bevan, 2003; Shedlock and Shen, 2003; Janssen et al., 2003; Bourgeois et al., 2002a). The role that CD4 T cells play in the CD8 secondary anti-LM response is not settled. Reports indicate that CD4 T cells are necessary only during the initial priming of

naïve CD8 T cells to differentiate into functional CD8 memory cells(Bourgeois et al., 2002a; Janssen et al., 2003; Shedlock and Shen, 2003; Marzo et al., 2004). In contrast, others show that CD4 T cell help for the CD8 memory response is essential and plays a role after the primary CD8 T cell response. Adoptive transfer of effector or memory CD8 T cells into CD4 T cell deficient mice demonstrates that CD4 T cells are required after, not during, the early programming phase(Sun et al., 2004). Furthermore, monitoring of the CD8 memory response with the IL-7Rα memory marker shows that a subset of CD8 T cells that appear early following LM infection express IL-7Rα^{hi}, and are responsible for generating a functional memory response (Huster et al., 2004). However, the generation of these IL-7Rα^{hi} cells and a memory response was impaired in absence of CD4 T cell help (Huster et al., 2004).

MHC class Ib H2-M3 responses have also been described in LM infection (Kurlander et al., 1992; Pamer et al., 1992). H2-M3 presents peptides that contain N-formylmethionine at the N-terminus (Lindahl et al., 1995). These peptides are short and hydrophobic and are released directly into the cytosol. In the cytosol they are proteolytically processed. H2-M3 restricted T cells are "promiscuous" in their recognition of peptides (Ploss et al., 2003). This may be a function of structural similarities of the defined epitopes (D'Orazio et al., 2003). H2-M3 restricted T cell responses peak on days 5 to 6 after primary infection, earlier than the MHC class Ia responses (Seaman et al., 1999). H2-M3 restricted T cells are cytolytic and produce IFN-γ. They have a role in the primary response to this bacterium as they appear early during the infection (Kerksiek et al., 1999). However, they make only a minor contribution to

the memory response (Seaman et al., 2000). One possibility is that H2-M3 restricted T cells have to compete with MHC class Ia restricted T cells and therefore they do not undergo clonal expansion (Hamilton et al., 2004). More recent studies examining the H2-M3 restricted T cell responses in spleen and lymph nodes during a secondary systemic infection find that the magnitude of H2-M3 restricted memory responses is down-modulated by increasing frequencies of MHC Ia restricted effector T cells (Ploss et al., 2005). Furthermore, while MHC 1a memory responses predominate in the spleen, H2-M3 restricted memory T cell responses remain prominent in the lymph nodes draining localized infections (Ploss et al., 2005).

III. Chronic Myelogenous Leukemia

The Oncology of CML

CML is a granulocytic cancer that results from the neoplastic transformation of a haematopoietic stem cell. It consists of two phases, the chronic and the blast phase. The initial chronic phase is characterized by the presence of the BCR-ABL oncogene, as well as a massive expansion of the granulocytic cell lineage. The median duration of the chronic phase is 3-4 years. The survival of chronic phase patients from diagnosis has a range of 1 to 20 years (Sokal et al., 1988).

With acquisition of additional genetic and molecular abnormalities, CML progresses from the chronic to blast phase. The additional mutations that contribute to the

progression of disease include mutations in TP53, RB and $p16^{INK4A}$ or overexpression of genes such as EVI1 and MYC (Faderl et al., 1999b). Moreover, additional CML chromosome translocations generating other fusion genes (such as AML1-EVI1) also contribute to advancement to the blast phase (Deininger et al., 2000a). Blast phase is characterized by a block of cell differentiation leading to the generation of 30% or more myeloid or lymphoid blast cells in peripheral blood, bone marrow or extramedullary organs. With progression into the blast phase, survival decreases and patients live only for a few months.

The Biology of CML

Although CML is granulocytic in nature, it is initiated by a malignant transformation in a self-renewing haematopoietic stem cell (HSC). HSCs can differentiate into granulocyte/macrophage progenitors (GMPs), megakaryocyte/erythtocyte progenitors (MEPs), or common lymphoid progenitors (CLPs). Since CML is a haematopoietic stem cell disease, it would be interesting to know if the oncogenic transformation affects other cells of the hematopoietic system. Recent studies in mouse models show that as CML evolves, normal hematopoiesis is suppressed as a result of competition for microenvironmental support, for example cytokines (Catlin et al., 2005). Moreover, lymphopoiesis, particularly the development of T cells, is compromised in that nonmalignant T-cell precursors can only be detected rarely (Takahashi et al., 1998). Furthermore, production of B cells from the neoplastic malignant clone occurs at low levels (Takahashi et al., 1998).

Evidence that CML arises from a pluripotent stem cell comes from studies using cytogenetic, fluorescence in situ hybridization (FISH) and X-linked markers (Gunsilius et al., 2000). While the leukemia initiating stem cell phenotype is very similar to that of normal hematopoietic stem cells - a low forward scatter (small size), $CD34^+$ (a stem cell marker), no lineage or activation markers (CD38 or HLA-DR) - there might be subtle differences not explored yet (Cicuttini and Boyd, 1994). The nonproliferating $CD34^+$ CML cells make up approximately 0.5% of the total $CD34^+$ compartment (Cicuttini et al., 1994). Relatively low numbers of such cells are able to engraft NOD/SCID mice and produce detectable leukemic progeny (Holyoake et al., 1999).

BCR-ABL Oncogene in CML

The majority of CML patients test positive for the BCR-ABL fusion oncogene in PCR diagnostic assays. Moreover, FISH diagnostic assays show the existence of a shortened chromosome 9 in patients. BCR-ABL is caused by the translocation t(9;22)(q34;q11), generating a shortened chromosome 9 termed 'Philadelphia chromosome'(ph). In this translocation, the ABL coding sequences upstream (5') of exon II on chromosome 9 are translocated to chromosome 22, and fused in-frame with the BCR gene downstream (3') of exon III. Depending on the precise breakpoints in the translocation and RNA splicing, different forms of BCR-ABL protein with different molecular weights (p185 BCR-ABL, p210 BCR-ABL, and p230 BCR-ABL) are generated in patients. These forms are associated with acute-lymphoblastic leukemia, CML and a milder form of CML, respectively. (Figure 4)

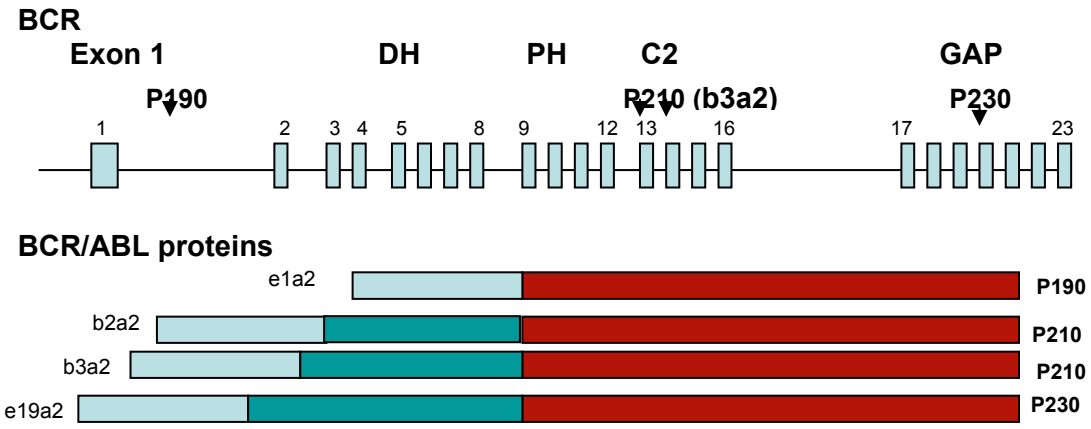

Figure 4. Representation of the *BCR* gene and Bcr/Abl translocation products. The 23 exons of the *BCR* gene (top panel) are shown with respect to the translocation breakpoints that give rise to the P190, P210 and P230 Bcr/Abl proteins.) The most common product of the Ph-translocation, P210, is found as two main variants that include what is commonly known as *BCR* exon b2 or exon b2 + b3 (exons 13 and 14). Above the exons, the approximate location of the different protein domains as encoded by the different exons is also shown. The Bcr/Abl fusion proteins (bottom panel) contain a constant Abl moiety encompassing exons 2-11 (red) and a variable Bcr part (blue (exon 1) and dark blue (variably exons 2-19)). P190 fusion proteins are generated as the fusion of exons e1a2, whereas the two common P210 variants are known as b2a2 and b3a2. A rare variant encoding a P230 is generated by an e19a2 fusion.

There are two components to the BCR-ABL fusion oncogene, BCR and ABL (Figure 5). BCR is a signaling protein in its nonmalignant form (Voncken et al., 1995a). BCR-deficient mice develop normally, although their neutrophils have been shown to produce excess levels of oxygen metabolites following their activation (Voncken et al., 1995b). ABL is a non-receptor tyrosine kinase that is expressed in most tissues (Woodring et al., 2003). ABL protein is distributed in both the nucleus and cytoplasm of the cells and can shuttle between the two compartments. It transduces signals from cell-surface growth factor and adhesion receptors to regulate cytoskeleton structure (Woodring et al., 2003; Hernandez et al., 2004). Mice with a homozygous disruption of the ABL gene are

variably affected, but mostly display an increased incidence of perinatal mortality, lymphopenia and osteoporosis (Schwartzberg et al., 1991). ABL-null mice are also smaller, with abnormal head and eye development (Tybulewicz et al., 1991).

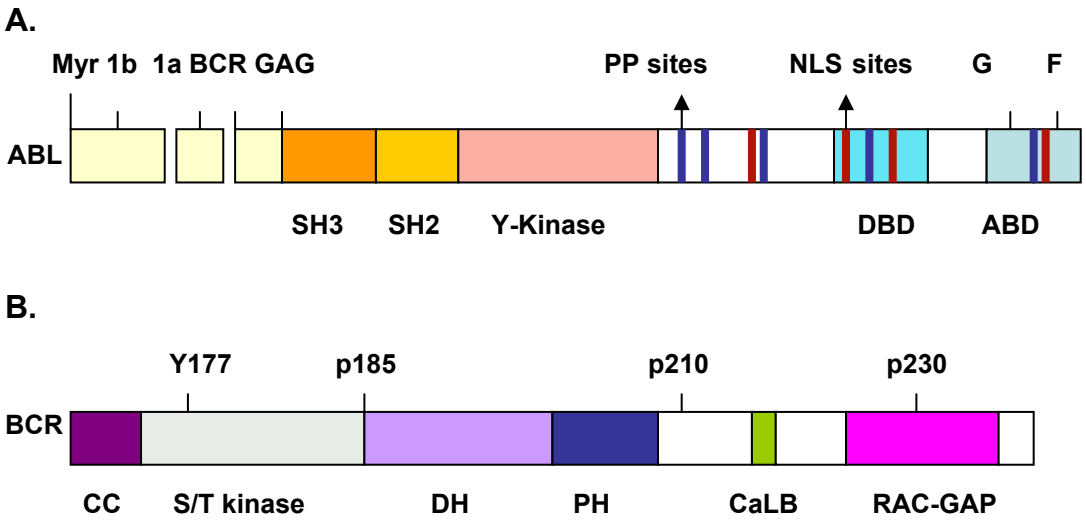

Figure 5. Several important domains make up ABL and BCR proteins. A. Two isoforms of ABL (human types 1a and 1b) are generated by alternative splicing of the first exon, one of them (1b) contains a myristoylation modification site (Myr). SRC homology 3 (SH3), SRC homology 2 (SH2), tyrosine-kinase (Y-kinase), proline-rich SH3 binding sites (PP), nuclear localization signal (NLS), DNA-binding domain (DBD), actin-binding domain (ABD), monomeric form of actin (G), filamentous form of actin (F), nuclear exporting signal (NES). B. BCR domains. Coiled-coil oligomerization domain (CC), serine/threonine kinase domain (S/T kinase), Dbl/CDC24 guanine-nucleotide exchange factor homology domain (DH), pleckstrin homology domain (PH), calcium-dependent lipid binding site (CaLB), RAC guanosine triphosphatase-activating protein (RAC-GAP).

During the translocation event that generates the chimeric BCR-ABL oncogene, BCR sequences fuse with ABL. This fusion brings new regulatory domains/motifs to ABL, such as the growth factor receptor-bound protein 2 (GRB2) SH2-binding site (tyrosine 177-Y177), thus increasing the tyrosine-kinase activity of ABL (Figure 5,6). Regions of BCR-ABL important in mediating oncogenic activities are the kinase domain, the oligomerization domains, the myristoyl-binding pocket, and the F-actin binding domain,

a determinant of BCR-ABL interaction with cytoskeletal components (Hantschel and Superti-Furga, 2004; Hantschel et al., 2005).

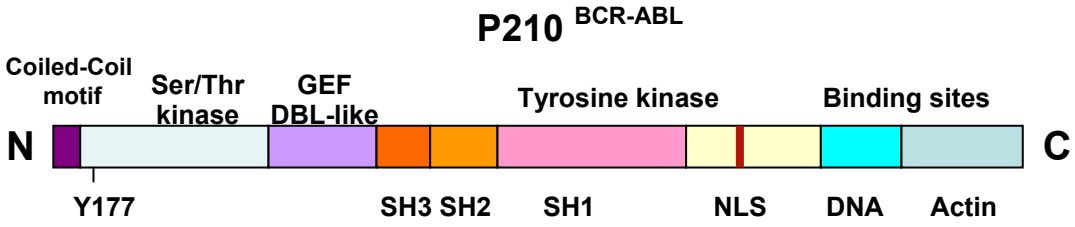

Figure 6. Functional domains of $p210^{BCR/ABL}$. GEF (guanine-nucleotide exchange factor), NLS (nuclear localization signal). For abbreviations refer to figure 5.

Many other signaling proteins, as well, interact with BCR-ABL and become disrupted through its oncogenesis (Figure 7). BCR-ABL transmits its signals through functional domains/motifs (i.e.: GRB2, CRKL, CRK, SHC, 3BP2, ABL-interacting proteins 1 and 2 and CRK-associated substrate). Other signaling proteins become phosphorylated in BCR-ABL expressing cells – for example: CRKL, CRK, SHC, docking protein 1, GAB2, STAT5, p85 subunit of PI3K, phopholipase Cγ, synaptophysin, VAV1, RAS GTPase activating protein, focal adhesion kinase FES, paxillin and talin (Deininger et al., 2000a). These proteins in turn activate a range of signaling pathways that activate proteins such as RAS, PI3K, AKT, JNK, SRC family kinases, protein and lipid phosphatases, and their respective downstream targets. In turn transcription factors such as the STATs, nuclear factor-κB and MYC are activated (Wong and Witte, 2004). The result of the signaling disruption by BCR-ABL oncogensis is an increase in proliferation and a decrease in apoptosis and cell adhesion. These events lead to an increase of the malignant cell population in the bone marrow and blood.

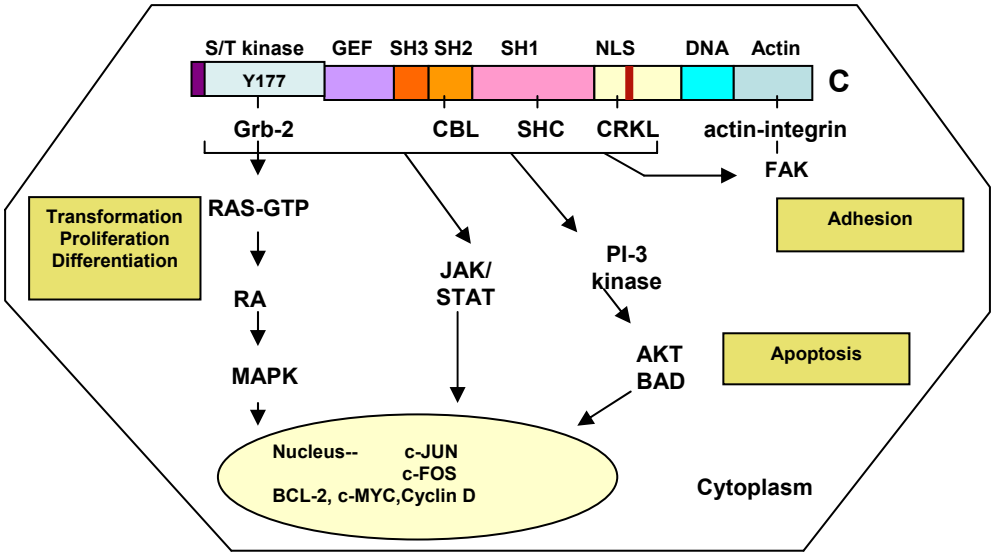

Figure 7. Signaling pathways of $p210^{BCR/ABL}$. Activation of RAS, Jak/Stat, PI-3 kinase pathways and focal adhesion complexes results in increased proliferation, differentiation, and decreased apoptosis and adhesion to the bone marrow stroma of the CML progenitors.

The BCR-ABL oncogene is essential for initiation, maintenance and progression of CML. This implies that BCR-ABL constitutes a good target for therapy in CML, however, an understanding of its essential domains is crucial to developing drug therapies. In order to gain an understanding of the important domains in BCR-ABL that cause transformation, various mutant forms of BCR-ABL have been expressed in mice. Mice that express a kinase deficient form of BCR-ABL, that is a mutation in the ATP-binding site of ABL, do not develop leukemia, even when the fusion protein is expressed in the long-term repopulating haematopoietic stem cell. This indicates that the ABL kinase activity is absolutely essential for BCR-ABL leukaemogenesis *in vivo* (Zhang and Ren, 1998). This result is also consistent with the finding that the kinase activity of ABL is required for BCR-ABL-mediated transformation in cultured cells (Ramaraj et al., 2004).

In order to study the transforming potential of BCR-ABL expression, several *in vitro* cell culture models have been established. Expression of BCR-ABL transforms established mouse fibroblast lines, factor-dependent haematopoietic cell lines and primary bone marrow cells (Ren, 2002). Expression of BCR-ABL in human $CD34^+$ primary cells causes increased proliferation in response to growth factors, increased growth-factor-independent survival, reduced adhesion to fibronectin and reduced chemotaxis to stroma-derived factor-1α (Ramaraj et al., 2004). Tissue culture and primary cells, however, are limited in representing the physiologically relevant target cells of BCR-ABL *in vivo*. The existence of unknown genetic abnormalities in established cell lines can also obscure the function of BCR-ABL. Therefore, further efforts have focused on establishing murine models to better understand CML.

Murine Models of CML

To study *in vivo* pathogenesis of CML and identify targets critical for BCR-ABL leukemogenesis, several murine models have been established. These include (1) transgenic models, (2) an inducible transgenic model, (3) xenotransplantation models, and (4) a bone marrow transduction/transplantation model (BMT). In addition, although embryonic stem-cell approaches to producing knock-in CML mice have been investigated, they have not been successful (Era and Witte, 2000; Castellanos et al., 1997). The activated BCR-ABL tyrosine kinase was found to be toxic during embryonic development and its expression from the BCR promoter caused embryonic lethality (Castellanos et al., 1997; Heisterkamp et al., 1991).

Two transgenic models of CML have been investigated. In one, BCR/ABL is under the control of the mouse metallothionein promoter which is inducible with heavy metals, but at the same time is constitutively and widely expressed in transgenic mice (Honda et al., 1995; Voncken et al., 1995a). The p210 BCR-ABL transgenic founders and progeny predominately develop T and B cell leukemia/lymphoma. In another transgenic model, BCR/ABL is under the control of the tec gene promoter, a haematopoietic specific promoter encoding a cytoplasmic kinase (Voncken et al., 1992; Heisterkamp et al., 1990). Due to the phenotypes assessed in this latter model, it is unclear whether it represents an accurate model of CML.

Investigators have also studied inducible transgenic mouse models. A recent approach expressed BCR-ABL under the control of a tetracycline-responsive promoter. Transgenic mice with p210 BCR-ABL under the control of the tetracycline response element (TRE) were mated to "transactivator" mice with tTA under control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter/enhancer. This is known to express tTA in epithelial cells and bone marrow (Huettner et al., 2000). These mice develop a chronic phase CML-like disease with symptoms which include neutrophilia, leukocytosis and splenomegaly (Koschmieder et al., 2005). Some animals demonstrate a biphasic phenotype, consisting of neutrophilia and subsequent B-cell lymphoblastic disease, reminiscent of blast crisis.

Xenograft models of chronic phase human CML have been difficult to develop because of the persistence of normal hematopoietic stem cells in most chronic phase CML patients and the lack of methods to selectively isolate the rarer CML stem cells. To

establish a xenograft model, the human leukemic long-term culture-initiating cells are identified –usually from patients in blast phase- and transplanted into sublethally irradiated NOD/SCID and NOD/SCID-β2microglobulin$^{-/-}$ mice (Cesano et al., 1992; Sirard et al., 1996; Skorski et al., 1992). The phenotypes of progeny mice include, in part, enhanced myelopoiesis and generation of primitive ($CD34^{+}$) leukemic cells displaying an autocrine IL-3 and G-CSF phenotype. A major advantage of this model is the ability to work directly with primary CML cells from patients. The limitations, in part, are that engraftment appears to be extremely variable, and that it may not be an accurate recapitulation of the pathophysiology of human CML (Lewis et al., 1998; Wang et al., 1998).

In more recent studies, the bone marrow transduction/ transplantation (BMT) model is used frequently to delineate the molecular mechanisms of BCR-ABL and to test CML therapies. In the early models of BMT, mice that received bone marrow cells transduced with p210 BCR-ABL also developed other haematopoietic neoplasms such as B-cell acute lymphoblastic leukemia (B-ALL) and macrophage tumors (Elefanty et al., 1990). In a later BMT model, mice developed a myeloproliferative disorder (MPD) closely resembling CML (Elefanty et al., 1990; Daley et al., 1990; Kelliher et al., 1990). The most recent BCR/ABL retroviral bone marrow transduction/transplantation model is the most accurate and informative one. This system involves the transfer of the BCR-ABL gene into primary murine bone marrow cells *ex vivo* using a replication deficient ecotropic retrovirus, followed by transplantation of genetically modified cells into irradiated syngeneic or immunodeficient recipient mice (Kelliher et al., 1990; Daley et

al., 1990). Additional refinements to the original protocol increased the transduction efficiency of CML progenitors. These include, in part, pre-stimulation of murine bone marrow cells with cytokine before retroviral transduction, and use of murine stem cell virus (MSCV)-based retroviral vectors for expression of p210 (Ilaria, Jr., 2004; Kotani et al., 1994). With these additional refinements 100% of the mice developed CML-like disease with symptoms of peripheral blood, BM granulocytosis and splenomegaly (Li et al., 1999; Pear et al., 1998).

The advantages of the BMT model, in part, are that (1) it mimics the somatic mutation that occurs in oncogenesis, (2) it affects only a limited number of hematopoietic cells, and (3) it can be used in different genetic backgrounds. Disadvantages to this system are the variability that can occur in disease outcome, and the immunosuppressive nature of the disease. Since the recipient mice have to be irradiated, the influence of the host's immunity on the development of leukemia cannot be fully assessed. Despite these limitations, the BMT model is by far the most efficient, reproducible and accurate CML murine model. Thus, this model has been used to establish the causative role of BCR/ABL in CML, to identify those signaling pathways and regions of BCR/ABL critical for leukemogenesis, and to explore the limitations of targeted CML therapy.

Current Therapies for CML

The first report on CML therapy was published in 1973. The report indicated that it was possible to induce cytogenetic remissions in the chronic phase of CML with intensive chemotherapy and splenectomy in a significant fraction of the patients

(Dowling, Jr. et al., 1973). Subsequently it was shown that these early intensive treatment protocols with or without splenectomy resulted in only marginal or no prolongation of survival (Silver et al., 1999; Kantarjian et al., 1985). Later a number of differentiating agents capable of inducing myeloid cell differentiation (i.e.: retinoic acid, bryostatin and vitamin D derivatives) were shown to cause differentiation and growth inhibition of human CML *in vitro* (Aglietta et al., 1985; Lilly et al., 1990). However clinical trials combining chemotherapy and differentiation agents in CML did not result in major therapeutic advances (Faderl et al., 1999a). Currently, the therapies that have induced favorable outcomes in CML patients include: interferon-α (IFN-α), autologous bone marrow transplantation (BMT), allogeneic BMT, donor leukocyte infusions following BMT after relapse of CML, IM and the newer dual target tyrosine kinase inhibitors.

IFN-α or IFN-α and cytarabine (arabinosylcytosine, Ara-C) is a form of CML therapy whose development preceded that of IM. In patients with favorable prognostic indices, IFN-α can lead to cytogenetic remissions (Selleri et al., 1997). IFN-α must be injected and is very toxic. Many patients are unable to tolerate the unpleasant side effects for the long periods of treatment required to obtain durable responses. The mechanism by which IFN-α inhibits growth of normal and CML progenitor cells is uncertain. A number of possible mechanisms proposed include: affects on multiple gene transcription and protein phosphorylation events, activation of dendritic cells and affects on Fas-R-mediated induction of apoptosis (Choudhury et al., 1997; Galvani and Cawley, 1989). Combined treatment of IFN-α and cytarabine leads to a higher percentage of complete

hematologic and cytogenetic remissions compared to IFN-α (Guilhot et al., 1997). However, the combination therapy causes more toxicity.

Another therapy is autologous BMT. Autologous BMT refers to transplant of the patient's own bone marrow after *in vitro* treatment to eradicate BCR-ABL^+ progenitor and malignant cells (Ljungman et al., 2006). Patients without histocompatible donors are candidates for this therapy. Autologous BMT follows marrow ablative intensive chemotherapy and irradiation regimens. Various methods such as peptide based vaccines using b3a2 junctional peptides have been used to preferentially eliminate residual Ph^+ progenitor cells in the graft while sparing normal stem cells (de et al., 1995). However, it is still uncertain whether this therapy is sufficiently selective and reliable to eliminate all Ph^+ progenitors while sparing enough normal stem cells to permit successful grafting.

Allogeneic BMT (also known as hematopoeitic stem cell transplantation-HSCT) following marrow ablative doses of chemotherapy and/or total-body irradiation is another treatment for CML (Bortin et al., 1992; Ljungman et al., 2006). Under the correct circumstances, allogeneic BMT can cure CML. However most patients are not eligible for this therapy because of advanced age or lack of a suitable stem-cell donors (Bortin et al., 1992). The success of allogeneic BMT depends on the generation of a graft versus leukemia (GVL) effect, but at the same time this success is limited by morbidity and mortality caused by graft versus host disease (GVHD). The exact mechanism of GVL is unknown. But GVL and GVHD are closely linked, and yet separable. Both GVL and GVHD are mediated by donor CD8 T cells and NK cells (Ljungman et al., 2006). While donor CD8 T cells recognize allogeneic and minor histocompatibility antigens on the

leukemic/host cells, NK cells respond to the lack of self inhibition on the leukemic/host cells. Host normal and leukemic cells lack the self-inhibitory molecules that the donor NK cells recognize; this leads to NK cell destruction of these cells. The difference between GVL and GVHD results from the specificity of these CD8 T cells and NK cells. In GVHD CD8 T cells respond to alloantigens on malignant and normal host cells. However, in GVL CD8 T cells respond to alloantigens on leukemic and normal hematopoietic cells. GVL results in destruction of host leukemic cells and remission of CML, while GVHD can result in death of the patient.

The GVL effect was first recognized in mouse models of stem cell transplantation in the 1950s (BARNES and LOUTIT, 1957). Recent studies further support the existence of the GVL effect. Due to the GVL effect, relapse rates are lower in those patients with significant GVHD, and the relapse rates are lowest if both acute and chronic GVHD develop (Horowitz et al., 1990; Weiden et al., 1981; Weiden et al., 1979). Moreover, use of T cell depleted bone marrow dramatically enhances relapse rates, further emphasizing the role of GVL effect (Apperley et al., 1988; Horowitz et al., 1990). In light of these observations, more recent work has focused on enhancing the GVL effect in relapse after allogeneic BMT. This is accomplished by transfer of donor leukocyte infusions (DLI).

DLI in patients with relapse after allogeneic BMT constitute another therapeutic approach to treatment of chronic phase CML (Kolb et al., 1995). DLI refers to transfer of either pretreated or untreated leukocytes. The source of DLI can be the patient's own bone marrow, or related or unrelated sources. The transfer of DLI to relapsed patients increases the GVL effect which leads to CML remission. In the early studies some of the

DLI caused severe or fatal GVHD (Collins, Jr. et al., 1997; Sehn et al., 1999). More recently, however, improved results have been obtained with $CD8^+$ depleted $CD4^+$ DLI ($CD4^+$ DLI). $CD4^+$ DLI are thought to act by inducing host-reactive cytolytic $CD8^+$ donor T cells to directly or indirectly inhibit the Ph^+ progenitors or stem cells (Zorn et al., 2002). Presently, persistent remissions can be obtained in 70-80% of DLI-treated chronic phase CML patients who relapsed after allogeneic BMT (Marks et al., 2002).

IM is another treatment for CML which has proved to be very successful. IM is given orally, and is mildly toxic. It has a more rapid hematologic response as well as a higher cytogenetic response than IFN-α (Eberle et al., 1995). IM is administered alone or in combination with other drugs (Holyoake et al., 1999). In addition, it is also used after allogeneic BMT to suppress relapse. More recently, IM is administered at higher concentrations (800mg/kg) than originally proposed in patients.

Although relapse risk remains low for chronic-phase CML patients who achieve a complete cytogenetic remission, relapses are frequent in advanced disease (Calabretta and Perrotti, 2004). For this, new ABL kinase inhibitors have been studied and two have reached clinical trials: AMN107 and dasatinib (BMS-354825). AMN107 is an IM analog with about a 30-fold greater potency against BCR-ABL and most IM resistant mutants *in vitro* (Weisberg et al., 2005). AMN107 is currently in clinical trials (phase II) to determine its effectiveness for treating IM-refractory CML. Dasatinib is a SRC/ABL kinase inhibitor that exhibits about 300-fold higher potency than IM against BCR-ABL and most IM resistant mutants *in vitro* (O'Hare et al., 2005; Shah et al., 2004). Whereas IM binds to a unique inactive conformation of ABL kinase, dasatinib is thought to bind to

the active conformation. The active conformation is more structurally conserved between ABL and SRC kinases than is the inactive form (Nagar et al., 2002). This enables successful inhibition of most IM-resistant mutants; however, it also reduces the specificity of the inhibitor and expands the profile of targets to include SRC family members (O'Hare et al., 2004; von et al., 2003).

Immune Response against CML

Immune surveillance against cancer was postulated many years ago. However, clinical and experimental evidence for a direct role of the immune system in protecting against spontaneous malignancies emerged slowly (Burnet, 1970). Recognition of tumor cells is commonly mediated by CTL and NK cells (Trowsdale, 2001). MHC class I molecules are important in tumor recognition in that they present peptide antigens to CD8 T cells (Garcia-Lora et al., 2003b). Tumors are thought to escape immune surveillance by MHC-class I downregulation/loss, downregulation of the tumor antigens, alteration of the apoptosis program expression of inhibitory cytokines or immunological ignorance (Garcia-Lora et al., 2003a; Garcia-Lora et al., 2003b).

Various clinical observations emphasize that the importance of immune surveillance mechanisms to control CML. For example, depletion of T cells in allogeneic SCT results in an increased probability of relapse (Drobyski et al., 1999). Likewise, relapse after allogeneic SCT can be cured with DLIs (van and Kolb, 1995; van et al., 1994). Antigen-specific leukemia-reactive T cells can be found in CML patients after successful treatment with IFN-α or allogeneic SCT (Horowitz et al., 1990; Kolb et al., 1995; Zorn et

al., 2002). As mentioned earlier, the therapeutic effect of allogeneic transplantation is thought to be due to GVL, the donor CD8 T cells and NK cells that recognize and effectively destroy leukemic cells. GVL is the desirable outcome in allogeneic transplantation for CML, while GVHD is the detrimental outcome. Evidence indicates that GVL is separable from GVHD. While significant GVHD occurs in 50% of patients treated with DLI, and disease response occurs in 90% of CML patients, 55% of patients who do not get GVHD also show disease response (Kolb and Holler, 1997; Giralt and Kolb, 1996).

Further evidence comes from studies that have identified specific BCR-ABL fusion region peptides that elicit CML-specific T-cell responses. BCR-ABL is present in nearly all Ph^+ CML patients. Therefore, it represents a potentially unique leukemia antigen. The most common transcript resulting from the BCR-ABL translocation is the chimeric mRNA b3a2 which is translated from the chimeric $p210^{BCR-ABL}$ (Figures 5 and 6). Translation of b3a2 mRNA results in the coding of a unique amino acid (lysine) within the fusion region. When the HLA-B8-restricted overlapping peptides inclusive of this lysine were pulsed onto HLA-B8 positive CML cells, some elicited T-cell proliferative responses (Bocchia et al., 1995; Dermime et al., 1995). Also, the b3a2-specific CTL generated *in vitro* from healthy individuals lysed CML cells (Dermime et al., 1995; Bocchia et al., 1996). Furthermore, in the peripheral blood of CML patients, b3a2 peptide/MHC tetramers have identified b3a2 specific CD8 T cells (Clark et al., 2001). Other BCR-ABL b3a2 peptides bind avidly to human HLA-A3(Clark et al., 2001; Norbury et al., 2000). In CML patients, circulating HLA-A3 BCR-ABL tetramer positive

cells that display an anti-CML cytotoxic response have been detected (Clark et al., 2001; Clark and Christmas, 2001).

Other CML tumor target antigens identified are derived from myeloid differentiation proteins associated with granule formation. One is proteinase 3 (Pr3), a neutral serine protease that is stored in primary azurophil granules and is maximally expressed at the promyelocyte stage of myeloid differentiation (Chen et al., 1994; Sturrock et al., 1992). Another is a myeloid-restricted protein, myeloperoxidase (MPO), a heme protein synthesized during very early myeloid differentiation (Borregaard and Cowland, 1997; Cowland and Borregaard, 1997). Both these proteins are over-expressed in 75% of CML patients (Dengler et al., 1995).

PR1 is an HLA-A2.1 restricted nanomer derived from Pr3 (Molldrem et al., 1999; Molldrem et al., 2000). After treatment of CML patients with IFN-α, use of PR1/HLA-A2 tetramers showed a significant correlation between cytogenetic remission and the presence of PR-1 CD8 T cells (Molldrem et al., 2000). PR-1 CTL have also been identified in the peripheral blood of allogeneic transplant recipients who have converted to 100% donor chimerism and therefore have achieved molecular remission. In addition, PR-1/HLA-A2 tetramer-sorted allogeneic CTL from CML patients in remission killed CML cells but not normal bone marrow cells (Molldrem et al., 2000). MY4 is a nine amino acid peptide derived from MPO that binds to HLA-A2.1, and can elicit leukemia-specific CTL (Molldrem et al., 2000; Bocchia et al., 1995). My4-specific CTL showed preferential cytotoxicity toward allogeneic HLA-A2.1^{+} myeloid leukemia cells (Molldrem et al., 2000).

Evidence indicates that BCR-ABL not only generates a CD8 T cell response, it also elicits a CD4 T cell response. Anti BCR-ABL CD4 proliferative responses have been observed in both murine models and normal human subjects. A 17-mer b3a2 fusion peptide generated a BCR-ABL-specific T-cell line that was HLA-DR2-restricted. This HLA-DR2-restricted T cell line proliferated in response to the peptide from healthy donors (ten Bosch et al., 1995). In addition, HLA-DR11 restricted CD4 T cell proliferative responses have been observed in normal donors using a 25-mer b3a2 junctional peptide (Bocchia et al., 1996).

Regardless of all this evidence for T cell responses against CML antigenic peptides, CML is able to evade the immune system and progress. This evidence, however, reinforces the major role that T cells play in control of a leukemic clone. In that, it is interesting to evaluate the effects of any drugs such as IM on the immune response. If IM influences the cellular immune branch, it could interfere with the ability of host T cells to control recurrent or residual tumor.

IV. Imatinib Mesylate

Development of IM

The discovery that BCR-ABL is required for the pathogenesis of CML, and that the tyrosine-kinase activity of ABL is essential for BCR-ABL mediated transformation, made ABL kinase an attractive target for therapeutic intervention (Zhang and Ren, 1998). In the early 1990s, a collaboration between Brian Druker's group at Oregan Health and

Science University and the scientists at Ciba-Geigy (now Novartis) searched for a small molecule that would inhibit the BCR-ABL tyrosine kinase activity. This collaboration discovered a phenylaminopyrimidine molecule, then called CGP 57148B. CGP 57148B, now called Gleevec or IM, is also known as STI571 (for signal-transduction inhibitor) and Glivec.

Beginning in 1998, promising laboratory results led to clinical studies and clinical trials. These clinical trials document the superior capacity of IM to rapidly reverse the clinical and hematological abnormalities of CML in chronic phase (Druker et al., 2001; Druker et al., 2002). In newly diagnosed chronic phase CML patients, IM induces complete cytogenetic response in more than 80% of patients. Patients with more advanced phases of CML also respond to IM, but this occurs much less frequently and treatment is less durable (Druker et al., 2002).

The Mechanism and Targets of IM

IM is a 2-phenylaminopyrimidine derivative with the molecular formula $C_{29}H_{31}N_7O{\cdot}CH_4SO_3$ and its relative molecular mass is 589.7. IM, a potent inhibitor of ABL tyrosine kinase, functions as a competitive inhibitor of ATP binding to the ABL kinase with inhibition constant (K_i) value of 85 nM (Cowan-Jacob et al., 2004)(Figure 8). The interaction of IM with the kinase domain involves about 21 amino acids (Nagar et al., 2002). It binds to a unique inactive conformation of ABL kinase, thereby making it highly specific (Nagar et al., 2002). The binding of IM to the kinase domain, therefore,

blocks access to ATP, preventing phosphorylation of any downstream substrate (Druker and Lydon, 2000).

IM inhibits all known forms of ABL including the normal ABL, BCR-ABL, v-ABL and c-ABL (Druker et al., 2001). IM inhibits proliferation and induces apoptosis in BCR-ABL$^+$ cell lines as well as fresh leukemic cells from Ph$^+$ CML patients (Deininger et al., 2000b; Deininger et al., 1997; Druker et al., 1996; le et al., 1999). In colony formation assays using *ex vivo* peripheral blood and bone marrow samples, IM shows inhibition of BCR-ABL positive colonies from CML patients. *In vivo*, it inhibits tumor growth of BCR-ABL transfected murine myeloid cells as well as BCR-ABL positive leukemia lines derived from CML patients in blast crisis.

IM also inhibits the receptor tyrosine kinases for PDGF (PDGF-R) and SCF (c-kit or CD117) and ABL-related gene (ARG) (Apperley et al., 2002; Nishimura et al., 2003; Okuda et al., 2001). *In vitro*, IM inhibits proliferation and induces apoptosis in gastrointestinal stromal tumor (GIST) cells, expressing the c-kit mutation (Wilson et al., 2005). IM is also active against macrophage colony stimulating factor receptor (c-fms) (Dewar et al., 2005a). Consequently, IM is used in treatment of CML, and has had favorable results in the treatment of GIST (c-kit mutation), myeloproliferative disorders (PDGF-R β mutation), eosinophilic disorders and mast cell disease (Pardanani et al., 2003a; Druker et al., 2001; Demetri et al., 2002; Apperley et al., 2002; Cools et al., 2003; Pardanani et al., 2003b).

The Limitations of IM

There are two major limitations to IM-based therapies for patients with CML. One is the persistence of BCR-ABL positive cells detected by a sensitive nested reverse-transcriptase PCR assay - this is known as residual disease (Bhatia et al., 2003). The other problem is the relapse of the disease due to the emergence of resistance to IM (Gorre and Sawyers, 2002).

Residual disease is caused by a persistent leukemia specific stem cell, a more primitive stem cell than the one IM can target. The leukemic stem cells in CML have a hierarchy, both short and long term repopulating cells (Hope et al., 2004). This stem cell hierarchy further complicates targeting the CML specific stem cell. Findings in CML patients indicate that the primitive stem cells are sensitive to the anti-proliferative effects of IM, but, unlike more mature proliferating Ph^+ cells, these primitive progenitors do not readily undergo apoptosis (Graham et al., 2002). High concentrations of IM, significantly higher than achievable *in vivo*, fail to eliminate the most quiescent cells. The quiescent leukemic stem cells also show resistance to several drug combinations including Ara-C with IM (Holyoake et al., 2001). While in the presence of IM one subset of primitive stem cells accumulates in G0/G1, another subset appears deeply quiescent and inherently resistant to IM. This latter subset cause persistence of leukemia specific stem cells. Suppression of CML therefore relies on continuous IM therapy.

Emergence of resistance to IM results from mutations in the BCR-ABL oncogene that impairs drug binding. The most frequent are point mutations in the kinase domain of the BCR-ABL gene (Gorre et al., 2001; Weisberg and Griffin, 2003). BCR-ABL kinase

mutations account for 60-90% of relapses (Cowan-Jacob et al., 2004). There are four distinguishable clusters affecting the BCR-ABL kinase domain. These mutations are in: (1) the ATP binding loop (P-loop), (2) T315, (3) M351, and (4) A-loop. T315 is the amino acid that contacts IM with a crucial hydrogen bond in the ATP pocket- the threonine is most frequently mutated to isoleucine (T315I) (Nagar et al., 2002) (Figure 8). M351 is the amino acid contacting the ABL SH_2 domain, an interaction that helps stabilize the autoinhibited conformation of ABL (Nagar et al., 2003). The A-loop is important because its position regulates kinase activity. Of these, the T315I mutation accounts for 10-15% of clinically observed mutations and confers complete resistance to all clinically available kinase inhibitors (Nardi et al., 2004).

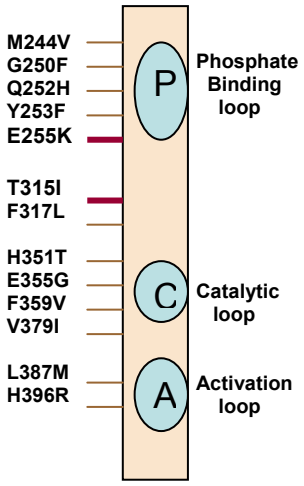

Figure 8. Point mutations in the Abl kinase domain. The BCR-Abl kinase domain, from amino acids 240–500, is shown with the adenosine triphosphate (ATP) binding loop (P), the catalytic loop (C), and the activation loop (A). The two most common mutations are designated in bold with a red line.

IM Therapy

IM is recommended for the treatment of newly diagnosed adult patients with Ph^+ CML in chronic phase, and for the treatment of patients with Ph^+ CML in blast crisis, accelerated phase or in chronic phase after failure of interferon-α (IFN-α) therapy. In addition, IM is recommended for the treatment of pediatric patients with Ph^+ chronic phase CML whose disease has recurred after stem cell transplantion or who are resistant to IFN-α therapy (Druker, 2002; Druker et al., 2002). IM is also administered after allogeneic bone marrow transplantation in candidate CML patients.

As with any drug, some adverse reactions are associated with IM, although the toxicity profile remains mild. Adverse reactions to IM in humans include diarrhea, edema, muscle cramps, musculoskeletal pain, nausea and vomiting and rash (Peng et al., 2004b). Myelosuppression is frequent, likely due to suppression of Ph^+ hematopoiesis. Generally, IM is well tolerated. However, some side effects may become apparent with longer follow-up. These late side effects in part include low testosterone levels, reduced sperm counts, and potential issues with pregnancies (Hensley and Ford, 2003).

Metabolism and Dosage of IM

IM is metabolized by a mitochondrial cytochrome P450 enzyme, CYP3A4. In humans, CYP3A4 forms the main circulating active metabolite of IM, a N-demethylated piperazine derivative. This metabolite shows in vitro potency similar to the parent IM. Elimination of IM is predominately in the feces, mostly as metabolites. Based on the recovery of compounds after an oral ^{14}C-labeled dose of IM, approximately 81% of the

dose is eliminated within 7 days. Native IM accounts for 25% of the dose, the remainder consists of metabolites (Peng et al., 2004a). The toxicity profile of IM is mild. IM is also a substrate for ABCG2 (ABC transporter G2), an active ABC transporter expressed on normal and leukemia stem cells (Burger and Nooter, 2004). IM undergoes active transport into CML cells via organic cation transporter type 1 (OCT1) (Burger et al., 2004). However, expression levels of ABCG2 and OCT1 on primitive CML stem cells have not yet been determined.

IM is administered at 400mg/day for patients in chronic phase, and 600 mg/day for patients in advanced phases (Druker et al., 2001). Recent clinical trials administer IM to patients in both phases of CML at 800mg/day. The primary goal of treatment with IM is to achieve a complete cytogenetic response (CCR)- that is to eliminate cells carrying Ph^+ cells (Peng et al., 2004b). Standard disease monitoring includes full blood counts, cytogenetics, and quantitative RT-PCR for BCR-ABL mRNA. These all are tailored to the level of response attained by a given patient. A hematologic response is defined as a 50% reduction in the white-cell count from baseline. A cytogenetic response is determined by the percentage of cells in metaphase that are positive for the Ph chromosome in the bone marrow. Molecular remission is defined as a negative BCR-ABL detection by RT-PCR.

At the standard dose of 400 mg/day IM used in CML, peak IM levels at steady state plasma levels are approximately 4.6μM with trough levels of approximately 2.13μM (Peng et al., 2004b). It has a $t_{1/2}$ of 19.3 hours, and its exposure is dose proportional for a dose range of 25 to 1,000mg daily (Peng et al., 2004b). This implies that administration

of a higher dose of IM results in a higher IM concentration in plasma, and therefore a higher body exposure to IM.

The pharmacokinetics of IM in humans indicate that IM is well absorbed after oral administration and the C_{max} is achieved within 2-4 hours post-dose (Nikolova et al., 2004; Peng et al., 2004a). Mean absolute bioavailability is 98% (Peng et al., 2004a). Following oral administration in healthy volunteers, the elimination $t_{1/2}$ of IM and its major active metabolite are approximately 19 and 40 hours, respectively. There is no significant change in the pharmacokinetics of IM on repeated dosing. The accumulation of IM is 1.5-2.5 fold at steady-state when IM is dosed once daily. At clinically relevant concentrations of IM, binding to plasma proteins in *in vitro* experiments is approximately 95%, mostly to albumin and α_1-acid glycoprotein (Gambacorti-Passerini et al., 2000).

Results of *in vitro* kinase assays on cell lines expressing constitutively active forms of ABL, Ph^+ cell lines derived from CML patients, and cell lines established from GIST patients indicate the inhibitory concentration (IC_{50}) value to be in the range of 0.1 to 0.5 μM. Exposure to IM leads to a block in proliferation and apoptotic cell death (Druker et al., 1996; Deininger et al., 1997). The effect of IM on normal hematopoiesis occurs at ≥ 1μM. Studies on IM inhibition of KIT proliferation reveal that the IC_{50} values are 0.1 to 0.3 μM in serum free media. But growth inhibition in serum-containing media requires higher drug levels (IC_{50} in the range of 5μM) (Druker et al., 1996).

IM and the Immune Response

IM affects many branches of the immune system. The cell types influenced by IM that have been investigated are $CD34^+$ progenitor cells, DCs, macrophages, NK cells and T cells. In this section, each of these categories will be discussed.

$CD34^+$ Progenitor Cells: IM displays a dose-dependent inhibition of growth on normal $CD34^+$ progenitor cells. This inhibition is not detected on the growth of stem cells. The effect on progenitor cells is independent of c-kit signaling and is linked to cell cycle activity. Furthermore, *in vitro* exposure of mobilized human $CD34^+$ progenitors to IM inhibits their differentiation into dendritic cells (Balabanov et al., 2005).

Dendritic Cells: IM affects both normal DC ($BCR\text{-}ABL^-$) and CML DC ($BCR\text{-}ABL^+$). Results on CML DC suggest that the numbers of DC in CML patients are reduced, and a high percentage of DC in CML patients are $BCR\text{-}ABL^+$ (Dong et al., 2003; Boissel et al., 2004). CML DC cells have defective antigen processing and migration patterns due to altered actin organization (Dong et al., 2003). They display decreased activity in MLR with allogeneic T cells (Wang et al., 1999). In addition, CML DC show a deficient expression of BDCA-4/neurophilin 1, a molecule involved in angiogenesis and DC-T-cell interactions, and administration of IM did not positively impact this change (Boissel et al., 2004). CML DC, when matured *in vitro* with cytokines (GM-CSF, IL-4), exhibit phenotypes similar to normal DC, and can induce a CML-specific primary T cell response (Eibl et al., 1997; Dietz et al., 2000).

Results on the effects of IM on normal DCs remain unsettled. While some data indicate that IM has positive immunomodulatory effects, other data indicate a negative

immunomodulatory effect. IM has been able to positively influence the functional APC qualities of normal DC, and result in overcoming tumor-induced CD4 T cell tolerance (Zeng et al., 2004; Sato et al., 2003; Wang et al., 2005). However, other data support an impairment of DC cell function, development and differentiation upon IM exposure. In the presence of 1-5μM IM, normal DC show reduced expression levels of CD1a and CD83, as well as HLA class II and costimulatory molecules CD80 and CD40 (Appel et al., 2004). These DC are unable to elicit primary T cell responses as well as T cell responses to recall antigen *in vitro*, perhaps due to a lower level of expression of costimulatory molecules. DC have an impaired responsiveness to maturation stimuli and a reduced immune stimulatory capacity. This effect is partly mediated through the nuclear factor κ_B/ RelB signal transduction pathway (Appel et al., 2005). Additionally, IM impairs the FMS-like tyrosine kinase 3 ligand (Flt3L)-mediated DC expansion and abrogates protective anti-tumor immunity (Taieb et al., 2004). The differences between these results could be due to exposure time to IM. The results showing a negative immunomodulatory effect use a longer interval of IM treatment in the range of 10-16 days, while the results demonstrating a positive immunomodulatory effect use shorter periods, in the range of 24 hours.

Macrophages: IM inhibits the *in vitro* development of monocyte/macrophage lineage cells from normal human bone marrow progenitor cells (Dewar et al., 2003). IM at therapeutic concentrations not only inhibits the maturation of monocytes into macrophages, it also inhibits their functional capacity (Dewar et al., 2005b). This includes phagocytosis of zymosan, IL-6 and TNF-α production in response to LPS, as

well as a decreased ability to stimulate a MLR (Dewar et al., 2005b). In addition macrophage colony stimulating factor (M-CSF) receptor, c-fms, is inhibited by IM, although this inhibition has a lower potency (IC_{50}= 1.42μM) than that observed for ABL (IC_{50}= 0.25μM), c-kit (IC_{50}= 0.1μM) or PDGF (IC_{50}= 0.25μM) (Dewar et al., 2005a; Buchdunger et al., 2000; Druker et al., 1996).

Natural Killer Cells: IM also interferes with NK cell interactions with DC in CML patients. In CML patients, about 73-98% of monocyte derived DC are CML DC (Eibl et al., 1997; Dietz et al., 2000). BCR-ABL translocation enhances NKG2D ligand on CML DC (Terme et al., 2005). Therefore CML DC have NK cell stimulatory capacity *in vit*ro, and this effect is counteracted with IM (Terme et al., 2005; Cebo et al., 2005). In normal individuals, IM inhibits NK cell functions through effects on the NKG2D molecule on NK cells (Cebo et al., 2006). In support of this, there is MICA expression on leukemic but not healthy donor $CD34^+$ cells. Treatment with IM, reduced MICA A/B protein levels on leukemic DC and $CD34^+$ cells, and consequently inhibited NKG2D mediated lysis by NK cells (Boissel et al., 2006a). In addition, small numbers of BCR/ABL^+ NK cells have been identified in the blood of patients with CML late in their disease course (Nakajima et al., 2002). BCR-ABL expression in NK cells alters their function and acquisition of killer immunoglobulin-like receptors (KIRS) *in vitro* (Chiorean et al., 2003). It also inhibits proliferation, and induces apoptosis in such $BCR\text{-}ABL^+$ NK cells (Chiorean et al., 2003).

T cells: In addition to the inhibitory effects of IM on $CD34^+$ peripheral blood progenitor cells (PBPC), monocyte-derived DC, monocytes, and NK cells, IM has been shown to

alter T cell responses. When stimulated through the T cell receptor (TCR) and CD28 or by mitogens or antigens, IM reduced the expansion of CD4 and CD8 T cells, and induced activation induced cell death (Cwynarski et al., 2004) (Dietz et al., 2004; Seggewiss et al., 2005). IM reduced the expression of CD25 and CD69 early activation markers (Seggewiss et al., 2005; Cwynarski et al., 2004). IM also reduced the production of IFN-γ from normal T cells (Aswald et al., 2002). In CD4 T cells from CML patients undergoing IM treatment, IM caused a reduction of IL-2 and T_{h1} cytokines (Gao et al., 2005). IM interferes with the TCR/Abl tyrosine kinase signaling pathway resulting in lack of phosphorylation on ZAP70 and reduced signaling through Erk (Zipfel et al., 2004; Gao et al., 2005). In addition, IM reduces the production of NF-κ_B in conjunction with a reduction in phosphorylation of LCK and ERK1/2 in stimulated T cells (Dietz et al., 2004). Moreover, IM affects c-kit signaling leading to deficits in pro T and B cell development (Agosti et al., 2004).

Research on the influence of IM on different immune cells indicate that IM affects the immune system. This influence could inhibit the immune response to tumors or pathogens, such as bacteria and viruses. Interference with immune response to pathogens can lead to an increase in pathogenic infections in patients who are treated with IM. In fact, there are case reports of increased infections in patients undergoing IM treatment (Ikeda et al., 2006; Lin et al., 2004). In addition, a reactivation of herpes zoster infection has been observed in patients treated with IM (Mattiuzzi et al., 2003). These observations suggest investigations on the effects of IM on immune cells merit further research.

Chapter Two

Objective

Imatinib Mesylate (IM) is effective at inducing complete cytogenetic remission in patients with chronic myelogenous leukemia (CML). IM is used increasingly more in treatment of other cancers, such as gastrointestinal cancers, in addition to its use after allogeneic bone marrow transplantation. Moreover, it is also administered at higher concentrations (800mg/day) to treat patients. While it is known that IM has immunosuppressive effects, the specifics are largely unknown. In particular, the influence/efficacy of IM on CD8 T cells is unknown. CML patients under treatment of IM have shown defects in DCs and macrophages. The effects of IM on T cells have only been tested *in vitro* utilizing human normal peripheral blood cells. These studies support the notion that IM has immunosuppressive properties on T cells. In light of an absence of data on the effects of IM *in vivo* on antigen-specific CD8 T cell responses, we used the adoptive transfer of OT-1 TCR Tg T cells into B6.Thy1.1 recipients in presence of a *Listeria monocytogenes* (LM) expressing the cognate epitope $OVA_{257-264}$ (LM-OVA). The primary goal of my thesis project has been to study the effects of IM on primary and secondary OT-1 antigen-specific CD8 T cells.

Current data testing the effects of IM *in vitro* indicate a dose dependent inhibition of proliferation and IFN-γ production, along with an induction of activation induced cell death in human peripheral blood T cells. Thus, the first specific aim of this project is to

determine what impact IM has on primary and memory CD8 T cells *in vitro*. The effects of IM on T cell expansion will be determined by *in vitro* CFSE dilution assays on primary and memory OT-1 cells activated in culture. Further studies will evaluate the apoptosis and intracellular IFN-γ production of these cultured OT-1 cells.

A more relevant question, however, is whether IM has any impact on the response of OT-1 T cells *in vivo*. The murine model of *Listeria monocytogenes* infection has been extensively used to characterize the contribution of CD8 T cells in immune protection. Thus, the second specific aim of my project will be to assess the effects of IM on the antigen-specific OT-1 primary response. The effect of IM on primary response of CD8 cells as well as on their function and expansion *in vivo* will be assessed. The phenotypic markers of survival such as IL-7Rα and activation such as CD122 will be monitored for any alterations. It is also known that CD4 T cell help is not necessary for generation of a primary response in an LM infection, but that it is needed after the primary response in order to generate an efficient memory response. It will also be of interest to determine if IM has any impact on the primary CD4 T cell response. The third specific aim of this project will be to determine if IM has an impact on memory antigen-specific OT-1 CD8 T cell response. This is an interesting question in that IM is increasingly used after allogeneic transplantation. Moreover, CML patients under treatment with standard or high doses of IM are already immunocompromised as a result of intense chemotherapy. The memory before and after recall response of antigen-specific T cells will be analyzed in immune B6.Thy1.1 animals. The functional and phenotypic characteristics of CD8 OT-1 cells, along with the CD4 T cell numbers and function will be assessed. Together,

these experiments should show the immunosuppressive effects of IM on CD8 T cells of cellular immunity, if any, along with any functional implications that IM may have (such as an increased susceptibility to recurrent infections). These studies will be significant not only for immunosuppressed CML patients undergoing therapy with the standard dose IM, but also for those patients in therapy with high dose IM. Moreover, the implications of these results will also be important for patients undergoing IM therapy after allogeneic transplantation, where graft-versus-leukemia (GVL) is the desired effect.

Chapter Three

Materials and Methods

Imatinib Mesylate. For *in vitro* cultures, 100mg tablets of IM (Gleevec; Novartis, Basel, Switzerland) were pulverized and dissolved in 10% DMSO at a 10mM stock and used at the indicated concentrations. Drug calculations were based on pure drug. For delivery to mice, *in vivo* stock of IM consisted of 100mg IM tablets dissolved in PBS or acidified water (pH 2.0) at a concentration of 8.9 mg/ml. Since the $t_{½}$ of IM in mice is ~ 8h, injection of 150µl of the *in vivo* stock intraperitoneally into each mouse corresponded to a dose of 75 mg/kg (Wolff and Ilaria, Jr., 2001). The mice received twice daily IM injections approximately 8h apart. Control animals received vehicle without drug. IM injections started on day 0 and continued daily through primary and secondary responses. For determination of the effect of IM on LM-OVA primary infection, IM injections started on day -4.

Mice. C57BL/6J (B6), C57BL/6.PL-Thy1^{a}/Cy (B6.Thy1.1) and OT-1 TCR transgenic mice (B6 background) were bred and maintained at the University of Texas Southwestern Medical Center animal facility under the approval of the Institutional Animal Care and Use Committee. OT-1 TCR transgenic mice were generated as previously described (Hogquist et al., 1994). C57BL/6.PL-Thy1^{a}/Cy (B6.Thy1.1) were described previously (Berg et al., 2003). All mice were used at 6-16 weeks of age.

Bacteria. LM-expressing full-length ovalbumin protein (LM-OVA) was originally provided by Dr. Hao Shen (University of Pennsylvania School of Medicine, Philadelphia, PA). Bacteria were grown on brain-heart infusion agar plates (BHI; Difco Laboratories, Detroit, MI) supplemented with 100μg/ml streptomycin (Sigma) (BHI-Strep). Virulent stocks were maintained by repeated passage through C57BL/6 mice. The LD_{50} dose of LM-OVA for B6 mice is approximately $2x10^4$ bacteria. For infection of animals, log phase cultures of LM-OVA were grown in 100μg/ml streptomycin supplemented BHI broth. The culture of LM-OVA was washed twice and diluted in PBS to the desired concentration (Pope et al., 2001). Bacterial numbers were determined by plating dilution of bacteria on BHI-Strep agar plates and incubating for 24 h at 37^o C. Colony counts from the plates were used to calculate the colony forming units (CFU)/ml.

Cell Culture and Reagents and *in vitro* CD8 T cell stimulation. Single cell suspensions were prepared from freshly isolated spleen or lymph nodes in HBSS supplemented with 5% FCS. Red blood cells were lysed in 1mM Tris Ammonium Chloride. After two washes in HBSS supplemented with 10% FCS, the lymph nodes were washed with and placed in PBS or HBSS at the desired concentration of $0.5x10^7$ cells/ml for intravenous injection into recipient mice. Only for *in vivo* CFSE proliferation assays a concentration of $2.5x10^7$ cells/ml was used.

For *in vitro* experiments, mouse splenocytes were cultured in 96 well plates at 0.5-$1x10^6$ cells/200μl of media in the indicated final concentrations of IM (0 μM, 2.5 μM, 5.0 μM, 7.5 μM, 10.0 μM, 15.0 μM, 50.0 μM). The cultures were grown in complete

RPMI 1640 (Life Technologies) supplemented with 10% FCS (Atlanta Biologicals), 25 mM HEPES, 2mM glutamine, 100U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20μM 2-ME and 10U/ml of IL2 (provided by Dr. Michael Bennett, UTSW) in the presence of 10nM SIINFEKL peptide (Peptide Synthesis Facility, UTSW). Control cultures that did not receive SIINFEKL were supplemented with 10 ng/ml of IL-7 (BD Biosciences) to promote cell viability. The cells were allowed to grow at 37^{o}C in humidified incubator containing 7% CO_2. After an overnight incubation, IFN-γ production was assessed, and after 3-4 days of growth, T cell expansion, and viability were assessed.

IM Measurement: Mice injected with IM were bled at 2 hours. Blood was collected in heparinized tubes and plasma was prepared by centrifugation. IM levels in plasma were quantitated following a liquid chromatographic-mass spectrometric assay (Dr. M.J. Egorin) (Parise et al., 2003).

Survival Studies: C57BL/6 mice were divided into 4 groups consisting of 3-4 mice/ group. Group 1 received no IM. Group 2 received 75 mg/kg of IM. Group 3 received 150 mg/kg of IM. Group 4 received 300 mg/kg of IM. The animals were monitored until death occurred.

Generation of LM-specific primary and secondary OT-1 cells: 1x10^6 lymph node (LN) cells from OT-1 TCR transgenic mice were injected intravenously into B6.Thy1.1

recipient mice, rested for 1-3 days, and infected intravenously with LM-OVA at a 0.1 LD_{50} dose ($2x10^3$ bacteria) to generate a primary response. LM-OVA immune animals that were infected >28 days previously were challenged with 1 LD_{50} ($2x10^4$) of LM-OVA to generate a secondary response. For analysis of antigen-specific OT-1 cells during the primary response, the blood or spleen were analyzed on day 7 of infection. For analysis of antigen-specific OT-1 cells during the memory response, the blood was analyzed on day 28. Antigen-specific OT-1 cells after recall were analyzed in the blood or spleen on day 6 of re-challenge.

Duration of IM delivery. The generation of a secondary OT-1 response was followed as described. The mice were divided into different groups consisting of 5 mice/ group. Group 1 received no IM throughout the entire experiment, while group 2 received IM throughout the experiment. Group 3 received IM during the first 7 days (primary response), and group 4 received IM after the first 7 days (after the primary response). Group 5 received IM up to day 28 (the day of re-challenge), and group 6 received IM after day 28 (after re-challenge). Group 7 received IM after the first 7 days, and IM injections were terminated before day 28 (before re-challenge). The data is presented as percentage control response at each of these intervals. It is also graphed as the duration of IM delivery referring to the total number of days each group received IM. The total number of days each group received IM are 7 days, 14 day, 28 days and 35 days.

Antibodies and cell staining: For flow cytometry the following antibodies or dyes from BD Biosciences were used: anti-CD8α (53-6.7), anti-CD90.2 (Thy1.2) (53-2.1 and 30-H12), anti-Ly5.2 (), anti-CD4 (RM4-5), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD122 (TM-β1), anti-CD16/CD32 (2.4G2), anti-CD94 (18d3), anti-IFNγ (XMG1.2), anti-AnnexinV, anti-BrdU mAb (clone B44) and 7AAD. Anti-CD127 (IL-7Rα) (A7R34) was obtained from eBioscience. Isotype controls for anti-CD127, anti-CD8α, and anti-CD90.2 were from BD Biosciences. Spleen ($2x10^6$) or blood cells were stained for 20 min on ice with the appropriate concentration of antibodies in PBS containing 2% FCS and 0.1% NaN_3 (FACS Buffer). Data were acquired using a FACScan or FACScaliber flow cytometer and were analyzed using CellQuest (BD Biosciences) or Flowjo (Treestar) software.

Blood Preparation For Flow Cytometry. Animals were bled via tail vein into HBSS 5% FCS containing heparin tubes. Red blood cells were lysed in 1mM Tris Ammonium Chloride and washed twice. Pellets were resuspended in FACS buffer and stained with the appropriate antibodies.

Detection of Apoptosis. The cultured cells were stained for cell surface markers and washed twice in PBS. $1x10^6$ cells were resuspended in 150μl of 1X Annexin V binding buffer. Annexin V and 7AAD was added for 15 min at room temperature in the dark. Another 150μl Annexin V binding buffer was added and the stained cells were analyzed

by flow cytometry within one hour. This protocol was modified from manufacture's protocol (BD Pharmingen).

***In Vitro* anti-CD3 Stimulation**. Purified anti-CD3ε (145-2C11) or isotype control IgG (G235-2356) were coated on 96 well tissue culture plates at 5μg/well in 100 μl PBS at 37^{o} C for 1 h. The antibody solution was removed and the wells were washed twice in PBS. The splenocytes were added at 1x10^{6} cells/well for an overnight culture. The next day the cells were stained with anti-CD4 and anti-IFN-γ as described.

Intracellular IFN-γ Staining. For intracellular staining (ICS), the splenocytes were cultured overnight with or without antigen. The next day cultured cells were treated with Golgi plug containing brefeldin A (BD Pharmingen) and harvested after 4 h. The cells were stained for cell surface markers, and fixed and permeabilized in Cytofix-Cytoperm solution (BD Pharmingen) for 20 min on ice. The cells were washed in perm/wash buffer (BD Pharmingen) twice and stained with anti-IFN-γ antibody in perm/wash buffer (Berg et al., 2002).

***In Vitro* CFSE Proliferation assays.** For *in vitro* assays, freshly isolated splenocytes were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) at a final concentration of 1μM for 1x10^{7} cells/ml OT-1 T cells at 37^{o}C incubator for 12 min. The cells were then cultured at 0.5x10^{6} cells/ well of 96 well plate and allowed to grow for 3-4 days at which time CFSE levels of OT-1 cells were analyzed by

flow cytometry. Calculation of percentage of cells in each round of division was performed according to an established protocol.

***In vivo* CFSE Proliferation assays**. For the *in vivo* CFSE transfer assays, single cell suspensions were prepared from freshly isolated OT-1 lymph node cells. Red blood cells were lysed in 1mM Tris Ammonium Chloride. The cells were labeled with CFSE at a final concentration of 10 μM for $1x10^7$ cells/ml of warm PBS containing 0.1% BSA at 37^oC incubator for 10 min (Lefrancois et al., 2003). The labeling reaction was quenched with RPMI containing 10% FCS, and the cells were washed and diluted at the desired concentration in PBS. Recipient B6.Thy1.1 mice received $5x10^6$ cells and a subsequent 0.1 LM-OVA infection. On day 3, donor CFSE-labeled cells were identified in the host spleen and analyzed for CFSE dilution by flow cytometry.

***In Vivo* CFU analysis of the effect of IM on primary LM-OVA infection.** IM treatment started on day -4. The control group received vehicle only delivery starting on day -4. Mice were infected with $2x10^3$ LM-OVA in 200ml PBS intravenously on day 0. On day 1 and 3 following infection, the spleens and livers were removed and homogenized in sterile water using a glass Dounce tissue grinder (Kontes). Serial 10-fold dilutions of the homogenate were plated in triplicate on BHI-Strep agar plates and incubated 24 h at 37^o C. Colony counts were averaged and corrected for dilution to yield the LM-OVA CFU per organ. The CFU limit of detection is 50 for the spleen, and 100 for the liver.

***In Vivo* CFU analysis of the effect of IM on LM-OVA memory response.** C57BL/6 mice received a primary sublethal i.v. infection of $2x10^3$ LM-OVA on day 0. IM injections started on day 0 and continued throughout the experiment. Control animals received vehicle only delivery. On day 28, animals received a secondary lethal re-challenge of LM-OVA at 50 LD_{50}. On day 1 and 2 following the secondary infection, spleens and livers were removed and the LM-OVA CFU per organ was determined as described. Controls included naïve animals that received 50 LD_{50} LM-OVA. Data is presented as log CFU protection in immune versus naïve animals. Log CFU protection was calculated by subtracting the average log bacterial burden in the spleens and livers of immune LM-OVA mice from the average log bacterial burden in the same organs of LM-OVA naïve mice.

BrdU labeling: For splenocyte labeling with BrdU (Sigma-Aldrich), naïve mice on day 0 and memory mice on the day of recall (day 28) were given BrdU (0.8 mg/ml) in the drinking water continuously for 6 days. Spleen cells were harvested and red blood cells were lysed with Tris Ammonium Chloride. After staining for the cell surface markers, the cells were fixed and permeabilized in Cytofix-Cytoperm solution (BD Pharmingen) for 20 min on ice. The cells were washed in perm/wash buffer (BD Pharmingen) twice and treated with 500 U/ml Deoxyribonuclease I (DNase 1)(Sigma) in PBS with 0.15M NaCl and 4.2mM MgCl at pH 5.0 for 30 min at 37^{o}C. The cells were washed and stained with anti-BrdU FITC in perm/wash buffer for 1 h at room temperature (Forster, 2004;

Tough and Sprent, 1994). The stained cells were analyzed by FACScalibur and CellQuest software.

Data Analysis: To compare results from multiple independent experiments, some data were normalized to the percentage of response observed in mice that did not receive IM. To establish this, the percentage of OT-1 T cells out of total CD8 T cells ($CD8^+Thy1.2^+$) in untreated animals from each experiment was averaged and set at 100%. Each point on the graph represents the response of OT-1 cells from individual animals relative to the control value for that experiment. Statistical significance was determined by Student's t-test, two-tailed distribution, and two-sample unequal variance as calculated by Excel program (Microsoft).

Chapter Four

Results

Effect of IM on OT-1 primary cells *in vitro*

Prior studies have shown that IM reduces proliferation but does not cause apoptosis of PBLs stimulated *in vitro* with PHA, Staphylococcal enterotoxin B (SEB) and anti-CD3 antibody (Seggewiss et al., 2005; Cwynarski et al., 2004). To investigate the effect of IM on CD8 T cell proliferation, we cultured naïve OT-1 T cells with their cognate antigen SIINFEKL in the presence or absence of IM for 4 days. In the absence of IM, about 80% of the cells divided, with 1-7 visible division cycles (Figure 1A). This pattern was also observed when IM was included in the culture at concentrations of ≤5μM. At 7.5-10μM of IM, cell division was reduced. At 15μM IM, only 10% of the cells divided and only one round of division was visible. In addition, the CFSE histograms also showed an inhibition of proliferation at 15μM IM (Figure 1B).

To determine whether IM induces apoptosis, we analyzed Annexin V expression in the cultured cells. After 4 days in culture in absence or presence of IM, the percentage of $7AAD^+$ only cells remained constant (~ 1%) (Figure 2A). The percentage of Annexin V and 7AAD double positive cells in absence of IM was 53%. This percentage increased at concentrations < 7.5μM IM (70%). Then at concentrations >7.5μM IM, the percentage of Annexin V and 7AAD double positive cells decreased to 31-49%. The percentage of single positive Annexin V^+ cells increased after 5μM IM (from 9% to

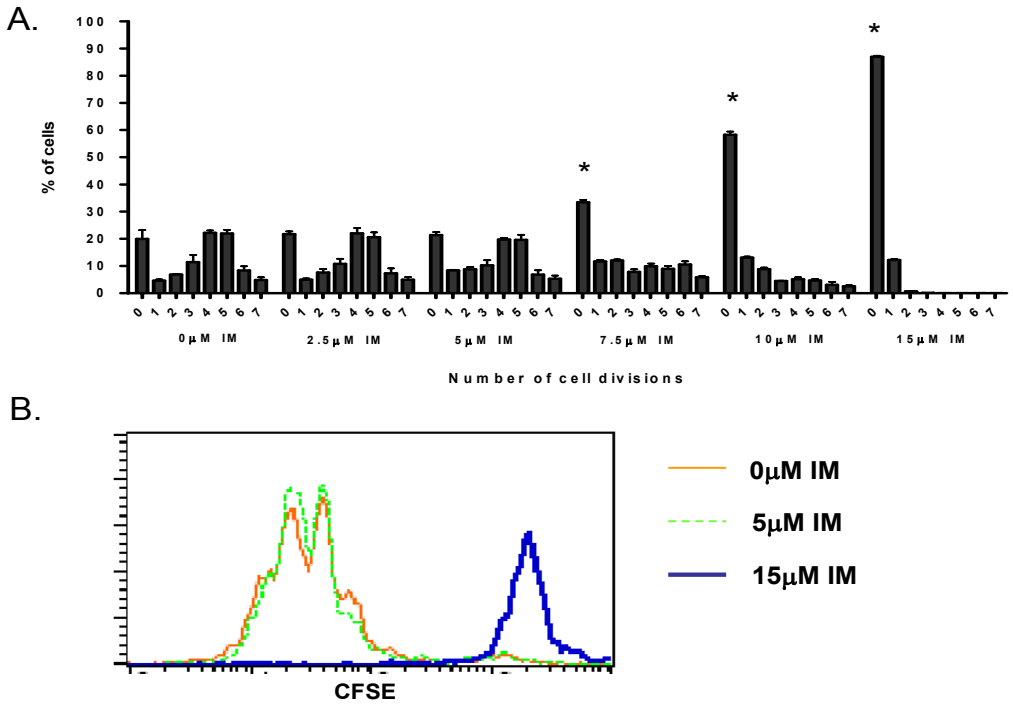

Figure 1. Effect of IM on primary OT-1 T cell proliferation. Primary OT-1 T cells were labeled with 1μM CFSE, incubated with the indicated [IM] in culture, +/- cognate SIINFEKL peptide for 4 days. A. Cell division of primary OT-1 T cells at indicated [IM] was determined by CFSE dilution on day 4. B. The histogram is representative data from CFSE dilution on day 4. Bars with * represent $p < 0.05$ compared to cultures receiving no IM. Data are representative of 3 independent experiments.

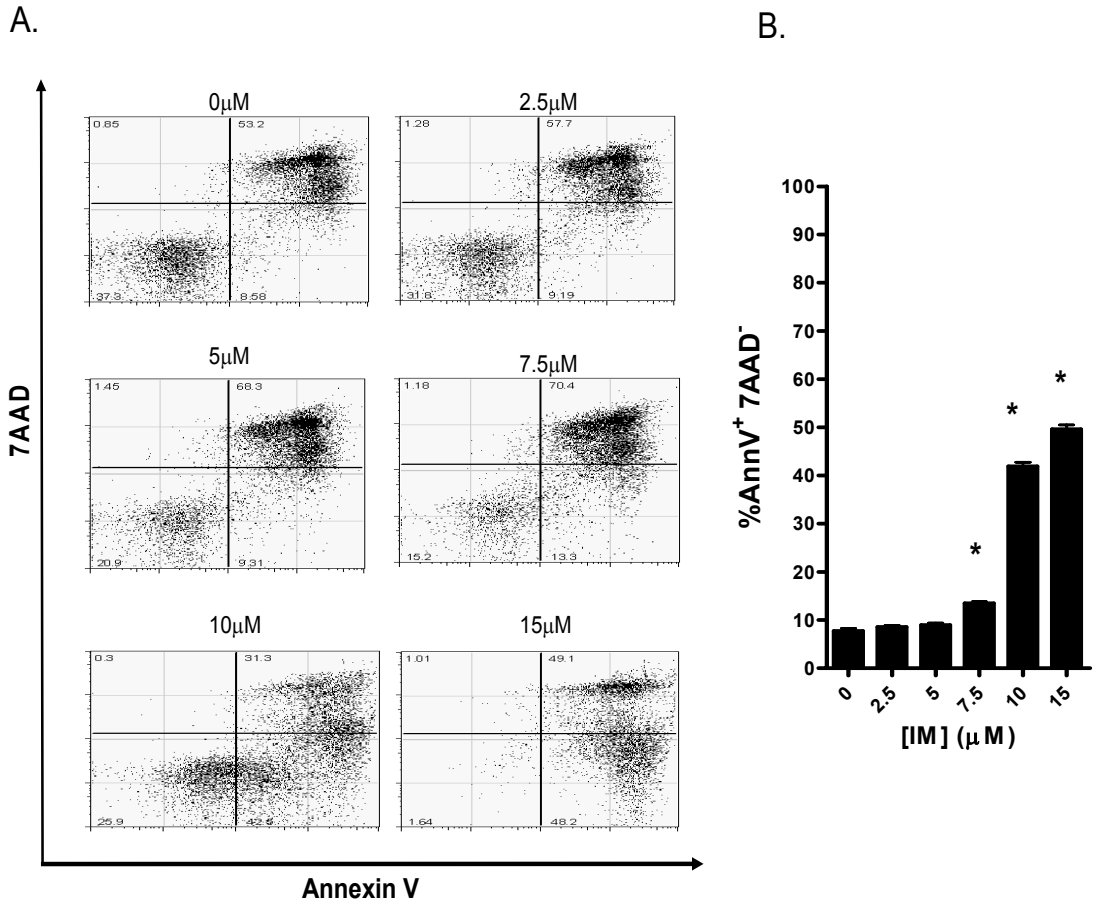

Figure 2. Effect of IM on primary OT-1 T cell apoptosis. Primary OT-1 T cells were incubated with the indicated [IM] in culture, +/- cognate SIINFEKL peptide for 4 days. A. The dot plots are representative data from Annexin V and 7AAD staining. B. Apoptosis of primary OT-1 cells was determined by Annexin V^+ and 7AAD staining. Bars with * represent $p < 0.05$ compared to cultures receiving no IM. Data are representative of 3 independent experiments.

48%). In the absence of IM or concentrations ≤5μM, ~7-9% of the cells stained with Annexin V only (Figure 2B). However, at 7.5μM IM, the cells underwent apoptosis, as detected by an increase in Annexin V single positive cells (13%). Apoptosis increased substantially as the dose of IM increased. At 15μM IM, about ½ of the cells stained with Annexin V. The remaining cells stained for both Annexin V and 7AAD.

The percentage of IFN-γ^+ cells was unaffected by doses of IM $\leq$5μM (Figure 3A and 3B). At 10μM IM, a reduction of IFN-γ^+ cells from 26% to 12% was observed (Figure 3A). At 50μM IM, there was complete inhibition of IFN-γ staining. The dot plots also show a reduction in IFN-γ^+ $CD8^+Thy1.2^+$ cells at 10μM (Figure 3B). Based on these results, IM did not affect cell division, apoptosis, or IFN-γ secretion of primary OT-1 cells until concentrations of >5μM were used.

Effect of IM on OT-1 memory cells *in vitro*

The effects of IM on antigen-specific memory CD8 T cells *in vitro* are unknown. The only experiment, which tests the effects of IM on target DCs, is a chromium release assay which uses effector memory CTLs derived from peripheral blood in response to a recall antigen (Appel et al., 2004). This experiment, however, does not investigate the effect of IM on memory CTLs. OT-1 memory cells were obtained from B6.Thy1.1 mice primed with LM-OVA >28 days previously. To determine the effect of IM on antigen-specific memory cells, memory OT-1 cells were cultured with IM for 3 days in the presence of SIINFEKL, and proliferation, apoptosis and IFN-γ staining were measured as described above. In the absence of IM, about 82% of the cells divided, with 1-7 visible

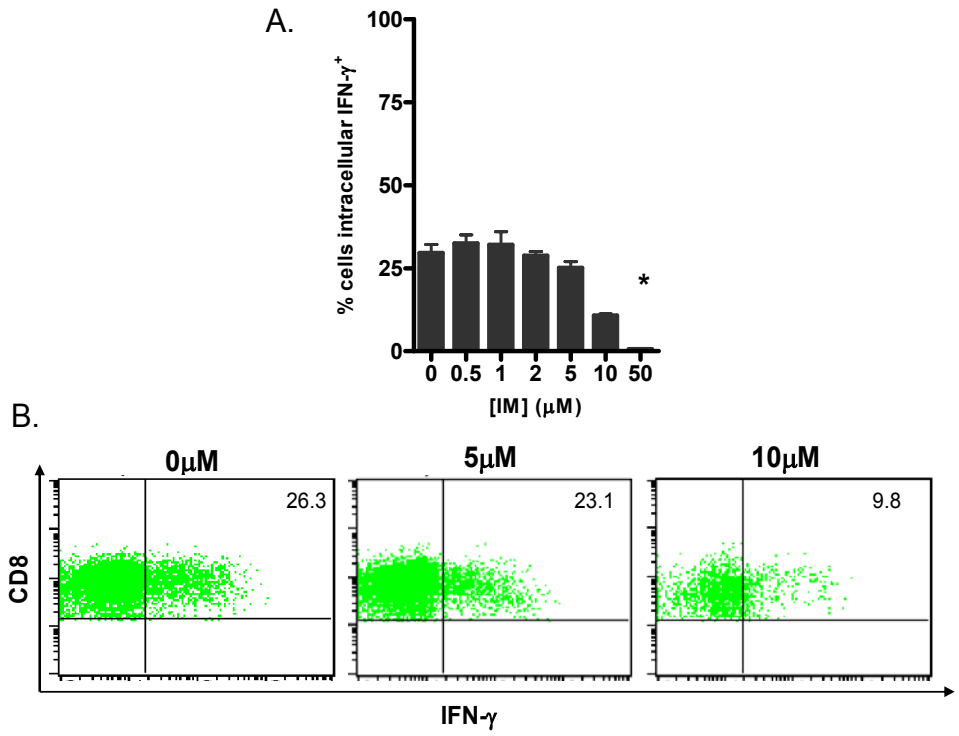

Figure 3. Effect of IM on primary OT-1 T cell IFN-γ staining. Primary OT-1 T cells were incubated with the indicated [IM] in culture, +/- cognate SIINFEKL peptide for 4 days. A. IFN-γ intracellular staining of primary OT-1 T cells was performed on day 4. B. The dot plots, gated on $CD8^{+}Thy1.2^{+}$ cells, are representative data from IFN-γ intracellular staining. Bars with * represent $p < 0.05$ compared to cultures receiving no IM. Data are representative of 3 independent experiments.

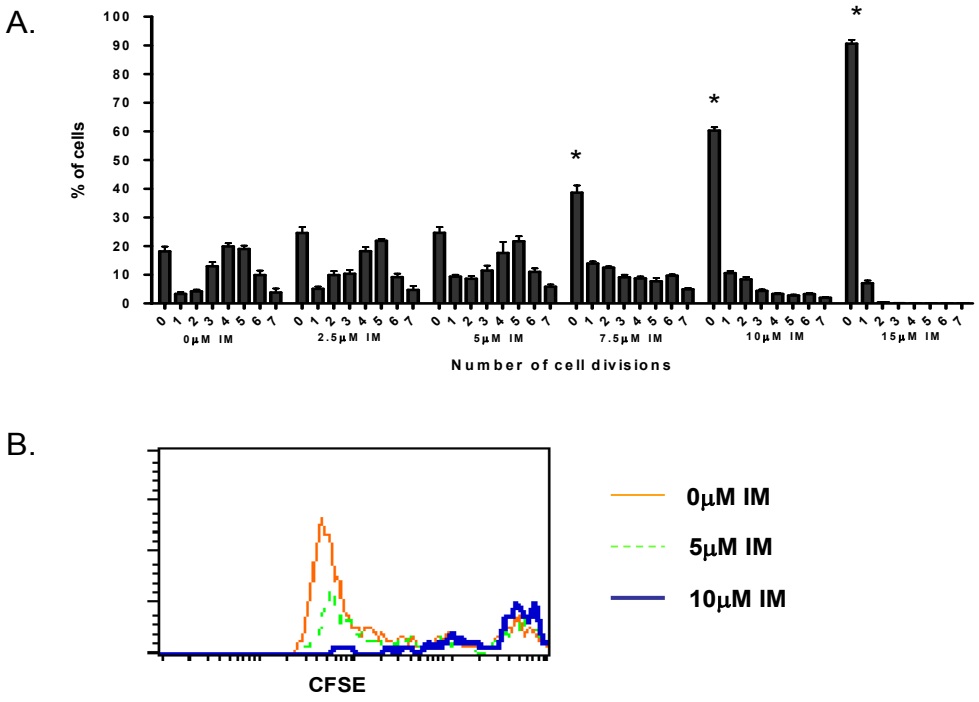

Figure 4. Effect of IM on memory OT-1 T cell proliferation. Memory cells were identified and gated as Thy1.2^{+}CD8^{+} cells in previously primed B6.Thy-1.1 recipients. Memory OT-1 T cells were labeled with 1μM CFSE , incubated with the indicated [IM] in culture, +/- cognate SIINFEKL peptide for 3 days. A. Cell division of memory OT-1 T cells at indicated [IM] was determined by CFSE dilution on day 3. B. The histogram is representative data from CFSE dilution on day 3. Bars with * represent $p < 0.05$ compared to cultures receiving no IM. Data are representative of 3 independent experiments.

division cycles (Figure 4A). This pattern was also observed when IM was included in the culture at concentrations of ≤5μM. At 7.5-10μM of IM, cell division was reduced. At 15μM IM, only 8% of the cells divided and only one round of division was visible. The CFSE histograms also showed a reduction in proliferation at 10μM IM (Figure 4B).

To determine whether IM induces apoptosis, we analyzed Annexin V expression in the cultured memory cells. After 3 days in culture in presence or absence of IM, the percentage of $7AAD^+$ only cells remained constant (~ 1.3%) (Figure 5A). The percentage of Annexin V and 7AAD double positive cells in absence of IM was 50.5%. This percentage increased at concentrations < 7.5μM IM (71%). Then at concentrations >7.5μM IM, the percentage of Annexin V and 7AAD double positive cells decreased to 30-45%. The percentage of single positive Annexin V^+ cells increased after concentrations of 5μM or greater was used (from 7.2% to 52.6%). In the absence of IM or concentrations ≤5μM, ~7-9% of the cells stained with Annexin V only (Figure 5B). However, at 7.5μM IM, the cells underwent apoptosis, as detected by an increase in Annexin V single positive cells (14%). Apoptosis increased substantially as the dose of IM increased. At 15μM IM, more than ½ of the cells stained with Annexin V. The remaining cells stained for both Annexin V and 7AAD.

The percentage of IFN-γ^+ cells was unaffected by doses of IM ≤ 5μM (Figure 6A and B). At 10μM IM, a reduction of IFN-γ^+ cells from 64% to 13% was observed (Figure 6A). The percentage of IFN-γ^+ cells also showed a reduction at 10μM IM on dot plots (Figure 6B). These results were similar to those seen with naïve OT-1 T cells in that

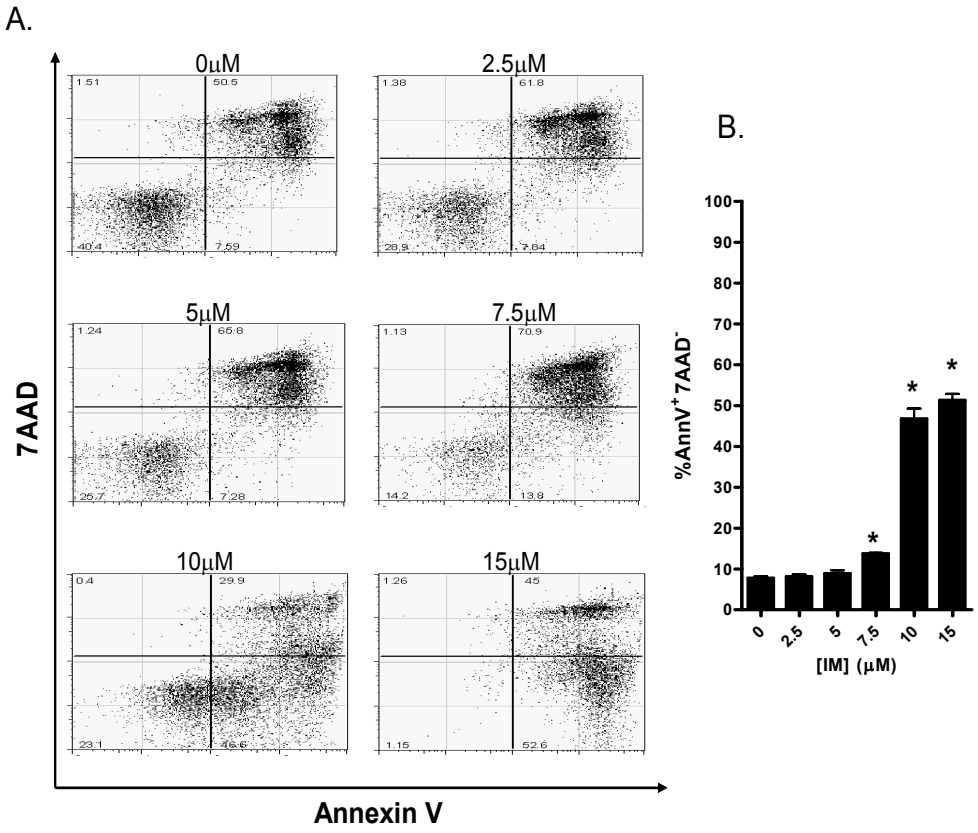

Figure 5. Effect of IM on memory OT-1 T cell apoptosis. Memory cells were identified and gated as Thy1.2^{+}CD8^{+} cells in previously primed B6.Thy-1.1 recipients. Memory OT-1 T cells were incubated with the indicated [IM] in culture, +/- cognate SIINFEKL peptide for 3 days. A. The dot plots are representative data from Annexin V and 7AAD staining. B. Apoptosis of memory OT-1 cells was determined by Annexin V^{+} and 7AAD staining. Bars with * represent $p < 0.05$ compared to cultures receiving no IM. Data are representative of 3 independent experiments.

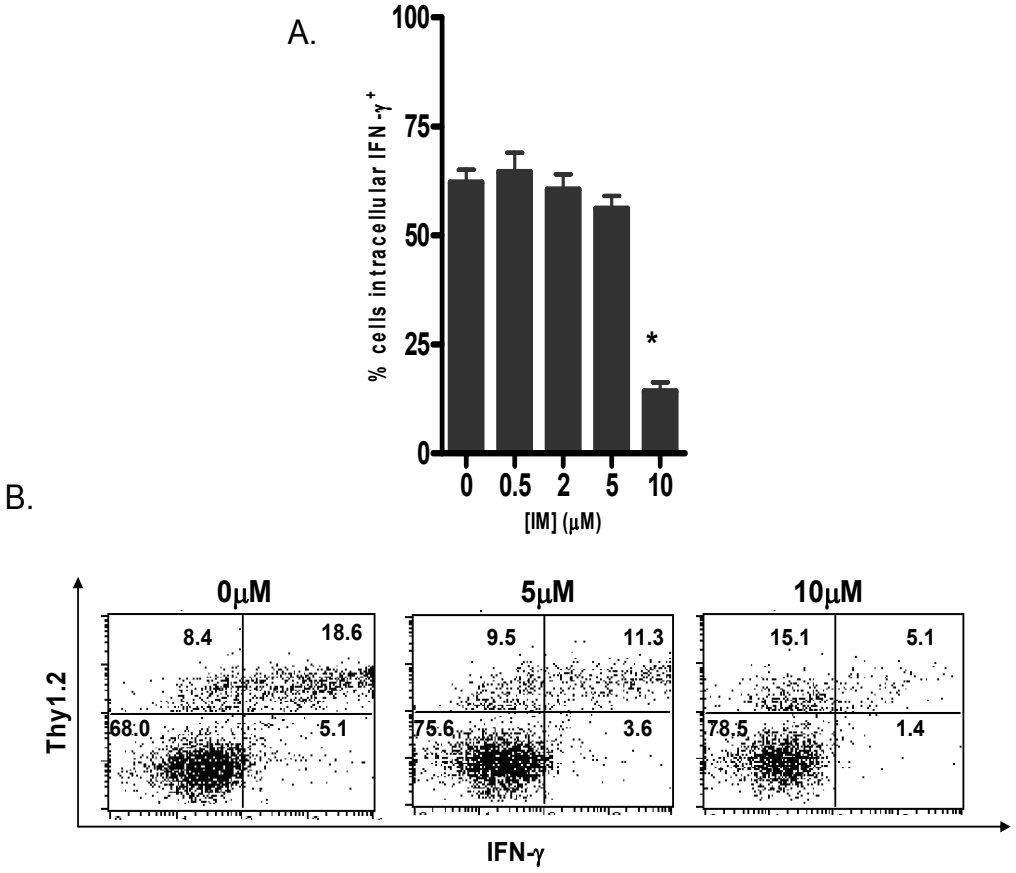

Figure 6. Effect of IM on memory OT-1 T cell IFN-γ staining. Memory cells were identified and gated as Thy1.2$^+$CD8$^+$ cells in previously primed B6.Thy-1.1 recipients. Memory OT-1 T cells were incubated with the indicated [IM] in culture, +/- cognate SIINFEKL peptide for 3 days. A. IFN-γ intracellular staining of memory OT-1 T cells was performed on day 3. B. The dot plots, gated on CD8$^+$ cells, are representative data from IFN-γ intracellular staining. Bars with * represent $p < 0.05$ compared to cultures receiving no IM. Data are representative of 3 independent experiments.

inhibition of proliferation, induction of apoptosis, and a decrease in IFN-γ staining were only noted at IM concentrations >5μM.

Effect of IM treatment on Survival of C57BL/6 Mice

Studies assessing the effect of IM in treatment or regression of CML in murine models use a range 50mg/kg to 150mg/kg of IM delivered either once or twice daily (Wolff and Ilaria, Jr., 2001; le et al., 1999). The CML murine models, including the bone marrow transduction/transplantation model, are not performed with mice on a C57BL/6 background (Zhang and Ren, 1998). Since our subsequent studies involve an *in vivo* determination of IM on the immune response, we determined a dose that would result in similar pharmacological plasma concentrations in B6 animals as those in BALB/C CML animals. B6 animals received 300mg/kg, 150mg/kg, 75mg/kg and 0mg/kg (vehicle delivery only). The animals were monitored until all the animals in high IM groups had died. In the 300mg/kg group, death occurred on days 2 and 3 (Figure 7). In the 150mg/kg group, mice started to die within 7 days of drug delivery, and were all dead by day 12. The control group and the 75mg/kg group of mice survived beyond 21 days of drug injections. Studies have used a concentration of 150mg/kg to retard CML in BALB/C mice (Wolff and Ilaria, Jr., 2001). Our data indicate that IM at 300mg/kg or 150mg/kg is detrimental to B6 mice with a morbidity rate of 100% by day 12. Therefore, the tolerated IM dose that we used in B6 mice is 75mg/kg IM.

Plasma Levels of IM Following Drug Delivery

IM is mainly metabolized by CYP3A4, a cytochrome P450 enzyme. Upon its breakdown, a particular pharmacological dose has to be reached in plasma before it can

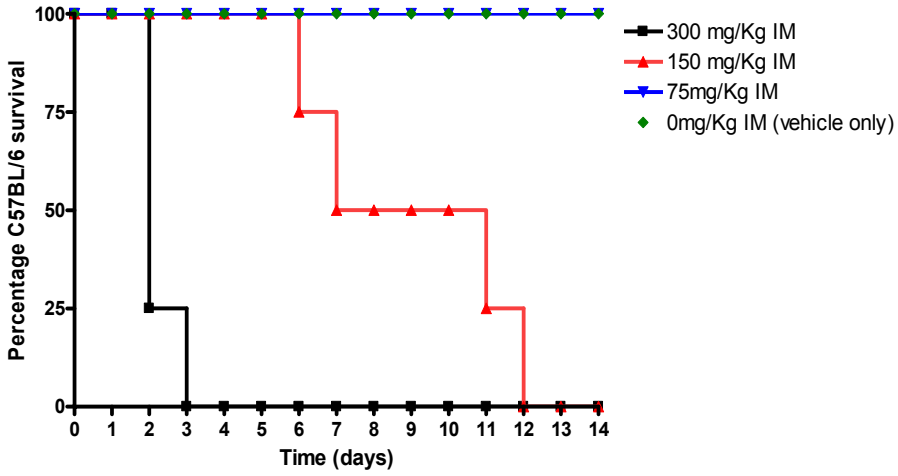

Figure 7. Survival of C57BL/6 mice with different doses of IM. C57BL/6 mice received twice daily intraperitoneal injections of 300mg/kg, 150mg/kg and 75mg/kg IM starting on day 0. A control group received vehicle only. The 75mg/kg and control vehicle delivery animals were monitored for 21 days. The data are from one experiment with 4 animals in each group.

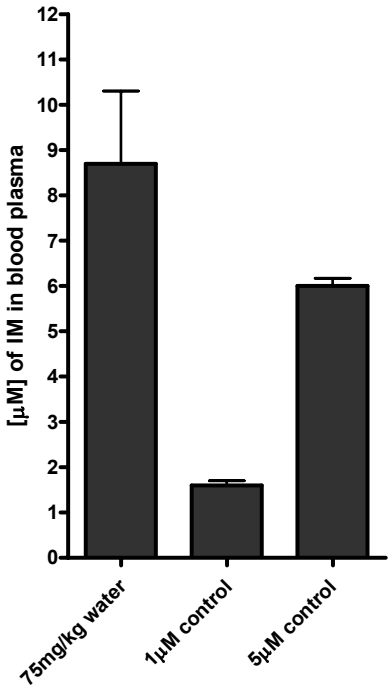

Figure 8. IM levels in blood. C57BL/6 mice received 75mg/kg of IM IP in acidified water. At 2 hours, the mice were bled, and IM levels in plasma were quantitated following a liquid chromatographic-mass spectrometric assay. Controls represent plasma spiked with 1μM and 5μM IM. The range of IM in plasma in the two experiments performed was 5.6 to 11μM (mean 8.7 ± 2.7). Data are representative of two independent experiments with 3 mice /group. Dr. M. J. Egorin's laboratory performed the liquid chromatographic-mass spectrometric assay.

block BCR-ABL activity in malignant cells. Clinical studies indicate a dose of 4.6μM IM is the effective pharmacological dose for tumor control and, this is reached at steady state by administration of 400 mg of drug to humans (Druker et al., 2001; Peng et al., 2004b). Similar doses, delivered either i.p. or orally, have been used in mice to control BCR-ABL tumors (le et al., 1999; Wolff and Ilaria, Jr., 2001). Recent clinical trials use a high dose of 800mg/kg of IM in patients (Kantarjian et al., 2004; Rea et al., 2006).

Mean IM plasma concentrations are dose proportional for the dose range of 25-1,000 mg (Druker et al., 2001; Peng et al., 2004b). This means that administration of a higher dose of IM will result in a higher concentration of IM in plasma. We determined if the delivery of 75mg/kg IM i.p. to B6 mice results in a pharmacological dose relevant for CML disease. After IM administration of 75 mg/kg twice daily, plasma levels measured in blood ranged from 5.6 to 11 μM (mean 8.7 ± 2.7) (Figure 8). These results suggest we achieved pharmacologically relevant IM concentrations in plasma.

Effect of IM on the growth of LM *in vivo*

Since the model employed measures the CD8 T cell response to LM-OVA, we investigated whether IM affects the growth of LM-OVA *in vivo*. Animals were infected with 0.1 LD_{50} LM-OVA, and CFUs were determined in the spleen and liver on days 1 and 3 (Figure 9). At the IM dose used (75 mg/kg), no effect on LM growth was detected in liver. In the spleen, IM had a small but significant effect on LM growth on day 1. However, this was not seen on day 3 (Figure 9).

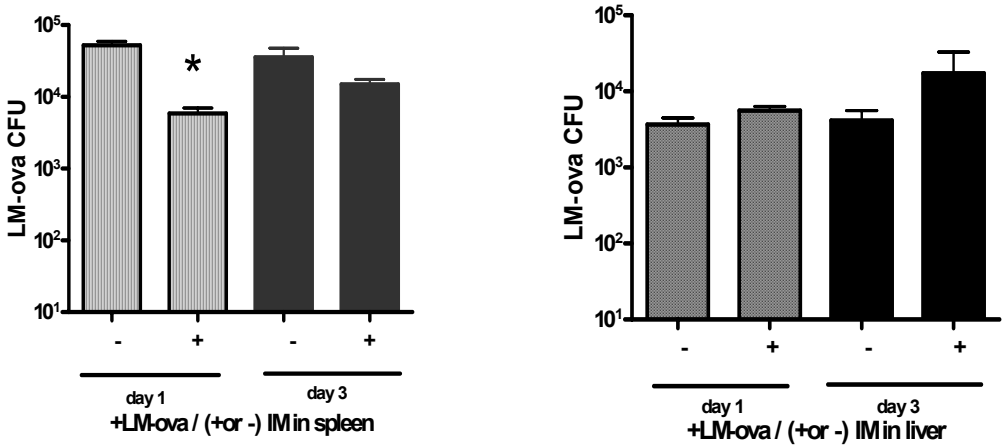

Figure 9. Effect of IM on listeriosis. C57BL/6 mice received 75 mg/kg of IM twice daily starting on day -4 and were infected with 0.1 LD_{50} LM-OVA. Spleens and livers were analyzed on days 1 and 3 after infection for LM-OVA colonies. Control mice did not receive IM. Data are representative of 3 independent experiments with 4 mice / group. Bars with * represent $p<0.05$.

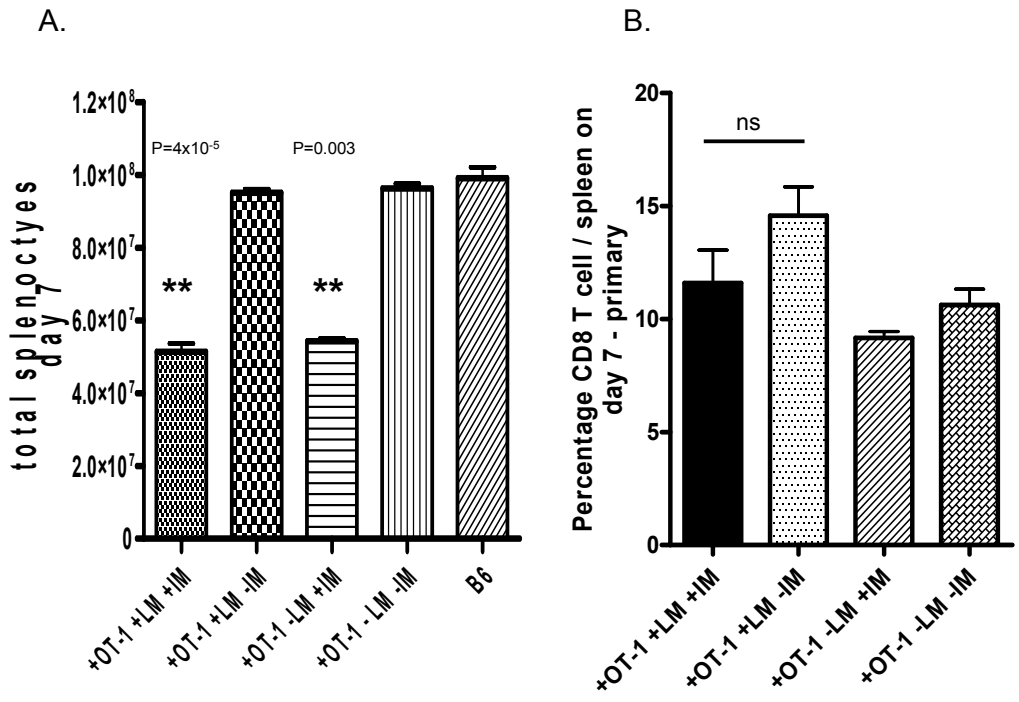

Figure 10. Total splenocyte counts and percentage CD8 T cells in spleen on day 7. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with daily intraperitoneal injections of 75 mg/kg IM twice daily. On day 7, the animals were analyzed for (A) spleen counts, and (B) for percentage total CD8 T cells by flow cytometry. The data are representative of two independent experiments with 4 mice / group. Bars with ** represent $p<0.001$.

Effect of IM on the primary response of OT-1 T cells *in vivo*

IM has been shown to affect immune responses against tumors as well as that of delayed type hypersensitivity (DTH) responses (Dietz et al., 2004). However, its direct effect on antigen-specific CD8 T cells *in vivo* has not been examined. B6.Thy1.1 recipients received 10^6 LN OT-1 T cells and 0.1 LD_{50} LM-OVA with or without daily injections of 75 mg/kg IM. On day 7, in presence of IM, the total splenocyte numbers decreased 2 fold (Figure 10A). This was seen both in presence and in absence of LM infection. However, the percentage of total CD8 T cells was not different between the IM-treated and untreated controls (Figure 10B). This indicates that the total number of CD8 T cells in spleen on day 7 is decreased.

When we gated on the percent of $CD8^+Thy1.2^+$ cells (Figure 11A), IM had no effect on OT-1 T cell responses in both spleen and blood on day 7. IM treated and control animals showed similar percentages of OT-1 T cells (Figure 11B). Considering that the total number of splenocytes decreased, this data indicates that IM delivery also decreased the total number of OT-1 T cells on day 7 in the spleen. To determine the functional status of these OT-1 T cells, we cultured the splenocytes from LM-OVA infected mice on day 7 with SIINFEKL for 18 hours, and measured IFN-γ by ICS. No difference was detected between animals receiving vehicle versus IM (Figure 12A and B).

Effect of IM on the primary OT-1 expansion *in vivo* during the primary response

The effect of IM on CD8 T cells can occur directly on OT-1 T cells, or indirectly through the effect of IM on host APC or CD4 cells. To determine whether IM acts on OT-1 donor or on host cells when treated for a relatively longer period (30 days), we

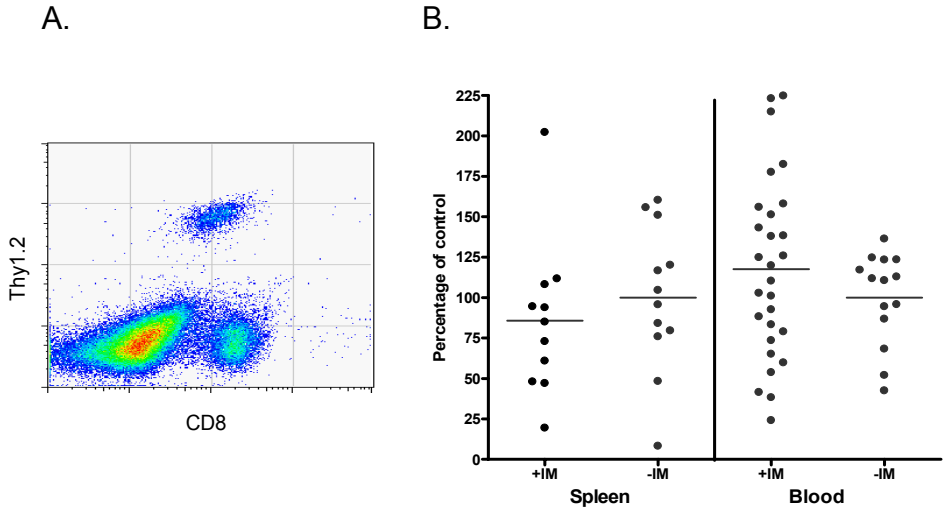

Figure 11. Effect of IM on the primary OT-1 T cell response *in vivo*. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with daily intraperitoneal injections of 75 mg/kg IM twice daily. A. On day 7, the animals were analyzed for donor OT-1 T cells, gated as $CD8^+Thy1.2^+$ cells, as shown in the dot plot. B. The graph represents percentage control response in PBL and spleen on day 7. To normalize responses between experiments, the percentage of OT-1 T cells out of total CD8 T cells ($CD8^+Thy1.2^+$) in untreated animals from each experiment was averaged and set at 100%. Each point represents the response of OT-1 cells from individual animals relative to the control value for that experiment. The percentage OT-1 T cells of CD8 T cells in control animals averaged 36%. Differences between -IM and +IM groups were not statistically significant (NS). Data are from 4 independent experiments.

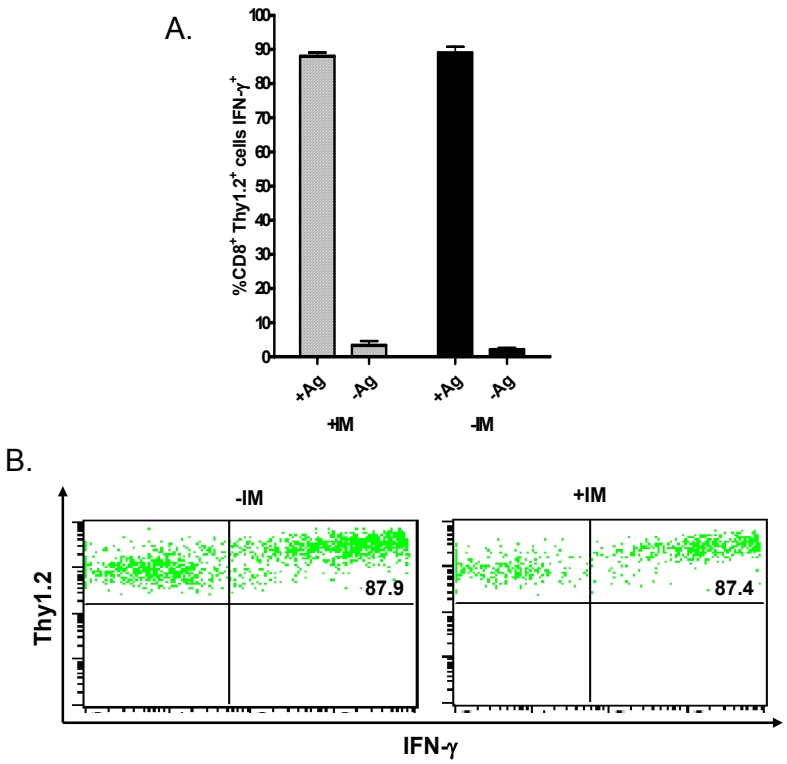

Figure 12. Effect of IM on the primary OT-1 T cell response *in vivo* as determined by ex-vivo IFN-γ staining. Splenocytes from primary mice (as described in Figure 11) were cultured overnight +/- cognate antigen and +/- IM, and 18 h later were stained for intracellular IFN-γ. A. The percentage of $CD8^+Thy1.2^+IFN\text{-}\gamma^+$ cells. B. The dot plots, gated on $CD8^+Thy1.2^+$, are representative data of IFN-γ^+ cells. Data are representative of 3 independent experiments with 4 animals in each group. The differences between the -IM and +IM groups are NS.

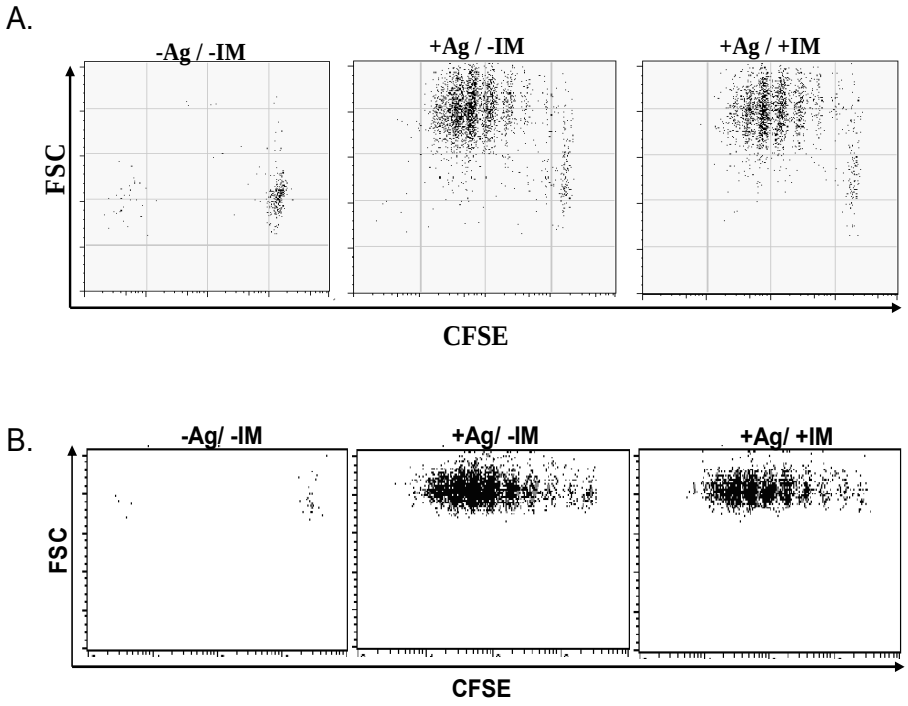

Figure 13. Effect of IM on the primary OT-1 T cell expansion *in vivo*. A. Donor OT-1 mice were treated with IM or vehicle for 28 days. LN cells were labeled with 10 μM CFSE, and $5x10^6$ cells were transferred to B6.Thy1.1 mice followed by a 0.1 LD_{50} LM-OVA infection. On day 3 of the response, spleens were analyzed for CFSE dilution and gated on donor $CD8^+Thy1.2^+$ T cells. A control group did not receive antigen (Ag). B. The hosts were treated with IM or vehicle for 28 days and then received a transfer of $5x10^6$ CFSE labeled OT-1 T cells along with 0.1 LD_{50} LM-OVA infection. A control group did not receive Ag. Data are representative of 3 independent experiments with 5 animals in each group. The differences between the -IM and +IM groups are NS.

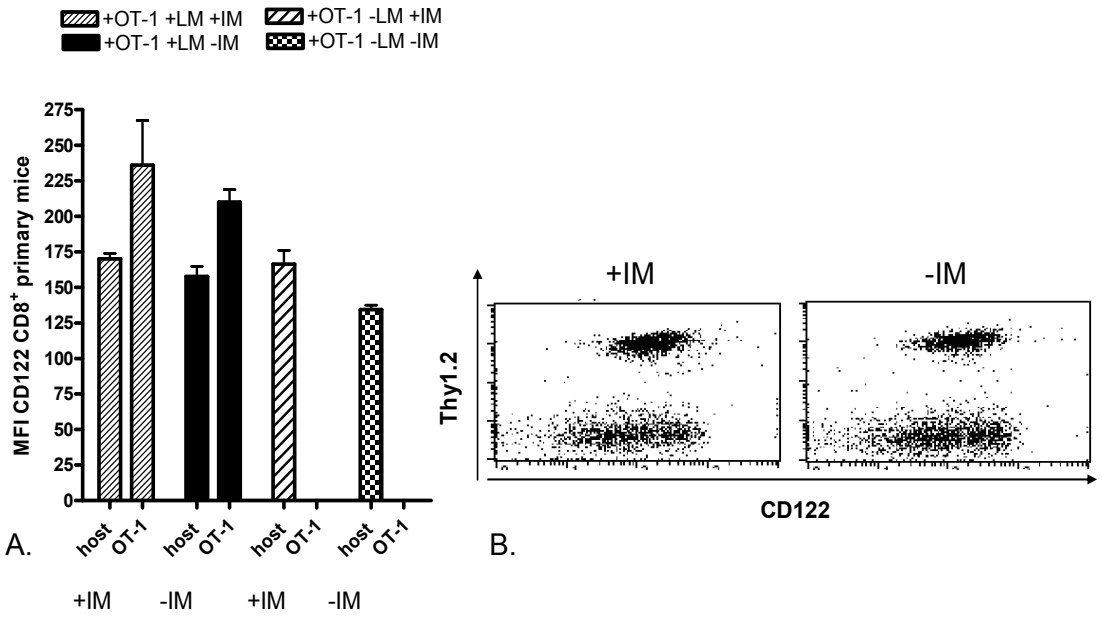

Figure 14. Effect of IM on expression of CD122 on OT-1 cells in primary response. Blood from IM treated and untreated animals that had received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA for a primary response, was analyzed on day 7 for CD122 by flow cytometry. The primary groups all received OT-1 cells (+OT-1). Two control groups did not receive LM-OVA (-LM), while one received IM (+IM), and the other did not receive IM (-IM). A. Mean fluorescence intensity (MFI) of CD122 on host $CD8^+$ and on $CD8^+Thy1.2^+$ cells. B. The dot plots are representative of $CD122^+$ on $CD8^+$ gate. The results are representative from 2 independent experiments with 4 mice in each group. The differences are NS.

measured the proliferative potential of IM-treated OT-1 T cells *in vivo* during the primary response. We pretreated OT-1 mice for 30 days with 75 mg/kg IM and then transferred CSFE-labeled OT-1 lymph node cells from pretreated mice into B6.Thy1.1 recipients. IM did not affect the extent of cell division of primary OT-1 T cells as displayed by CFSE dilution measured on day 6 (Figure 13A). When we pretreated Thy1.1 recipients for 30 days with 75 mg/kg IM and then transferred untreated CSFE-labeled OT-1 lymph node cells to these recipients, similar results were obtained (Figure 13B). These results indicate that, regardless of whether donor OT-1 cells or host cells had received IM treatment for 30 days, IM did not influence the expansion of primary OT-1 cells.

Effect of IM on expression of CD94 and CD122 during the OT-1 primary response

CD94 is a common marker for CD8 T cell activation (Gunturi et al., 2003; Moser and Loetscher, 2001). CD94 is expressed on memory and effector human CTL (Jabri et al, 2002). CD122 is the β chain of IL-2R. Naive T cells upregulate CD122 and CD25 (IL-2Rα) upon activation, respond to IL-2 and expand (Cantrell and Smith, 1983). To assess phenotypic changes on IM-treated primary OT-1 T cells, we analyzed the markers CD122 and CD94. The levels of MFI of CD122 on antigen specific cells remained unaltered in IM-treated animals in comparison with the controls (Figure 14A and 14B). The MFI of CD94 on OT-1 T cells was also unchanged during the primary response (Figure 15A and 15B).

Effect of IM on expression of IL-7Rα during the OT-1 primary response

Since expression of IL-7Rα, a marker for memory cells, early in the response identifies a distinct subset of memory T cells crucial for functional memory response, we

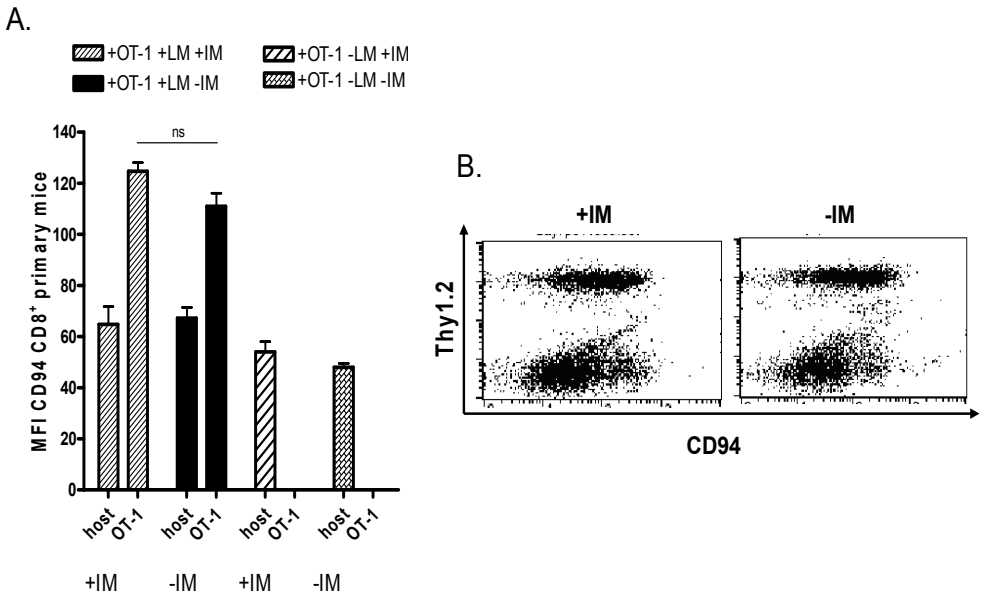

Figure 15. Effect of IM on expression of CD94 on OT-1 cells in primary response. Blood from IM treated and untreated animals that had received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA for a primary response, was analyzed on day 7 for CD94 by flow cytometry. The primary groups all received OT-1 cells (+OT-1). Two control groups did not receive LM-OVA (-LM), while one received IM (+IM), and the other did not receive IM (-IM). A. Mean fluorescence intensity of CD94 on host $CD8^+$ and on $CD8^+Thy1.2^+$ cells. B. The dot plots are representative of $CD94^+$ on $CD8^+$ gate. The results are representative from 2 independent experiments with 4 mice in each group. The differences are NS.

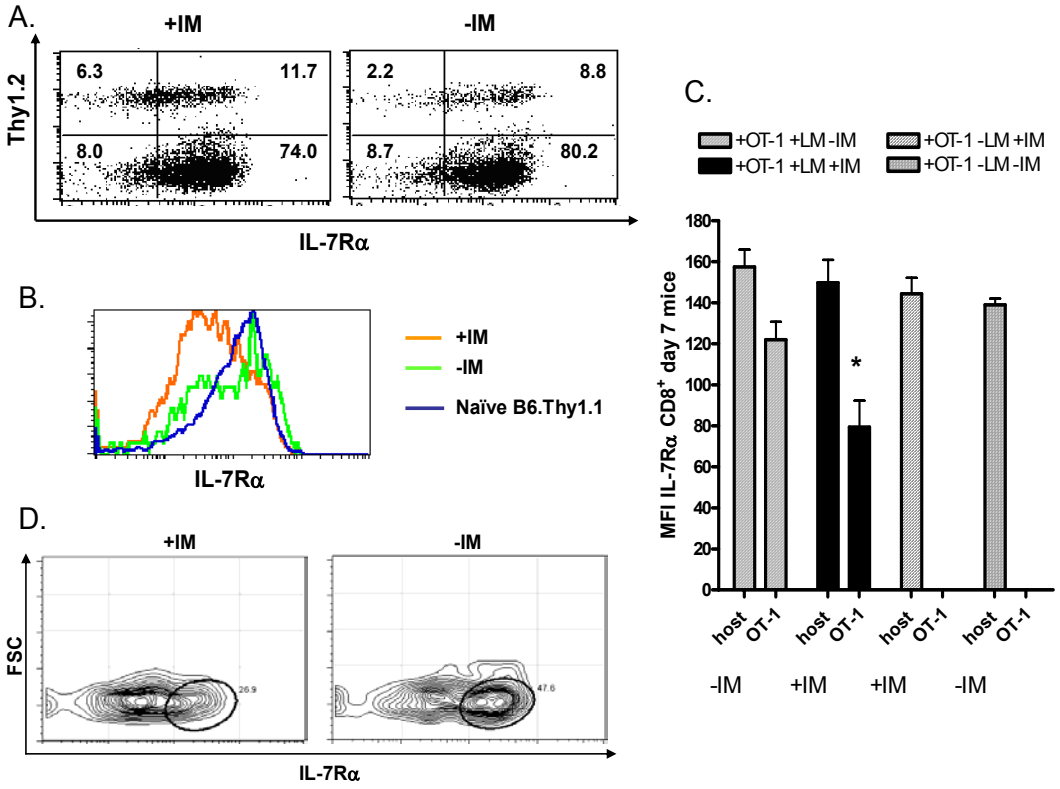

Figure 16. Effect of IM on expression of IL-7Rα on OT-1 cells in primary response. Blood from IM treated and untreated animals that had received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA for a primary response, was analyzed on day 7 for IL-7Rα$^+$ (CD127$^+$) by flow cytometry. A. Representative data, gated on CD8$^+$, shows percentage of IL-7Rα$^+$ on host and OT-1 CD8 T cells. B. Representative IL-7Rα histogram is gated on CD8$^+$Thy1.2$^+$ cells for the IM-treated and untreated controls. The naïve B6.Thy1.1 histogram is gated on CD8$^+$Thy1.1$^+$ cells. C. Mean fluorescence intensity of IL-7Rα on host CD8$^+$ and on OT-1 (CD8$^+$Thy1.2$^+$) cells. The primary groups all received OT-1 cells (+OT-1). Two control groups did not receive LM-OVA (-LM). One group of the -LM controls received IM (+IM), and the other did not receive IM (-IM). D. Representative data shows IL-7Rα^{hi} cells in the CD8$^+$Thy1.2$^+$ gate. In this experiment, the percentage CD8 T cells in PBL averaged 17.6% and percentage OT-1 of CD8 T cells averaged 3.5% in control animals. The percentage of IL-7Rα^{hi} expressing cells ranged from 26-48% in controls (mean 34.9± 9.2) and from 15-27% in IM-treated animals (mean 20.4± 4.7), ($p < 0.05$). The results are representative from 3 independent experiments with 4 mice in each group. Bars with * represent $p < 0.05$.

tested for IL-7Rα expression on OT-1 T cells on day 7 (Huster et al., 2004). Since the expression of IL-7Rα is high on naïve cells, a positive IL-7Rα gate was chosen according to IL-7Rα staining on CD8 T cells of naïve untreated controls. We noted that IM decreased the expression of IL-7Rα on OT-1 T cells (Figure 16A). The ratio of IL-7Rα^{+}/ IL-7Rα^{-} cells of OT-1 gate had a 2.1 fold reduction from the control to the IM treated group (Figure 16A). The fold difference in the ratios of IL-7Rα^{+}/ IL-7Rα^{-} cells of the host CD8 gate, from untreated controls to vs IM treated animals, remained similar. The histograms of IL-7Rα expression on OT-1 cells also showed a downregulation in the IL-7Rα expression (Figure 16B). Further, when the data was plotted as MFI of IL7Rα expression, a decrease was noted on OT-1 cells in IM treated animals compared to controls while expression on host CD8 T cells was equivalent in both IM and control groups (Figure 16C). We also identified primary OT-1 cells that expressed high levels of IL-7Rα (IL-7Rα^{hi}) early in the response by gating for IL-7Rα^{hi} population (Figure 16D). IM treatment decreased the expression of IL-7Rα^{hi} primary OT-1 cells from 47.6% (range 26.3-47.6%) to 26.9% (range 15.1-26.9%) (Figure 16D). These results indicate that IM did not affect the primary response as measured by the percentage of total CD8 T cells, percentage of OT-1 number of antigen-specific cells, IFN-γ secretion or expansion *in vivo*. In addition IM did not affect the expression of activation markers CD122 and CD94 on primary OT-1 cells. But IM decreased the number of total splenocytes, CD8 T cells, and OT-1 T cells. Furthermore, the percentage of OT-1 cells that expressed high levels of IL-7Rα also decreased.

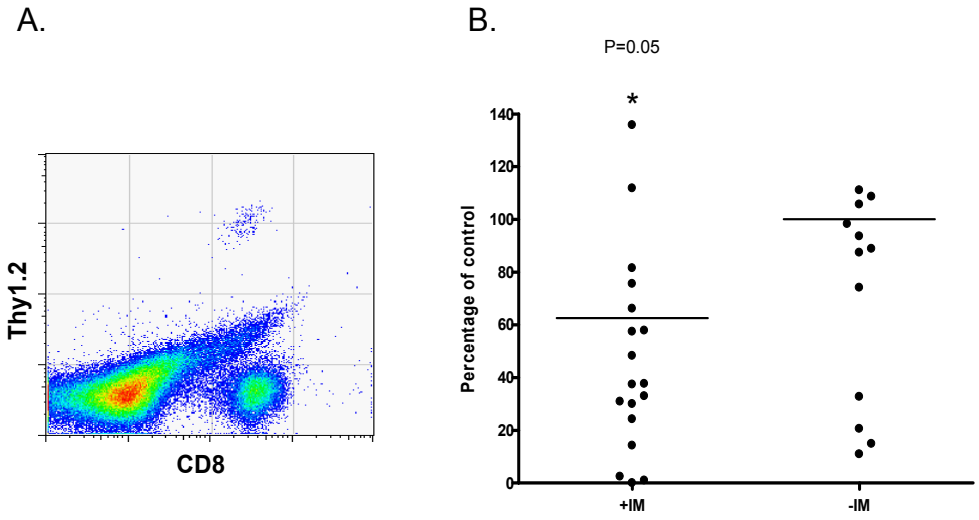

Figure 17. Effect of IM on OT-1 memory cells before re-challenge. Animals received OT-1 T cells and were infected with LM-OVA with or without twice daily injections of IM. On day 28, the blood was analyzed for the percentage of memory OT-1 cells in PBL. A. The dot plot represents donor OT-1 T cells, gated as $CD8^{+}Thy1.2^{+}$ on day 28. B. Percentage control response in PBL on day 28. Data were normalized as in Figure 11. The percentage of OT-1 of CD8 T cells in control animals averaged 9%. Data are from 4 independent experiments. Bars with * represent $p < 0.05$.

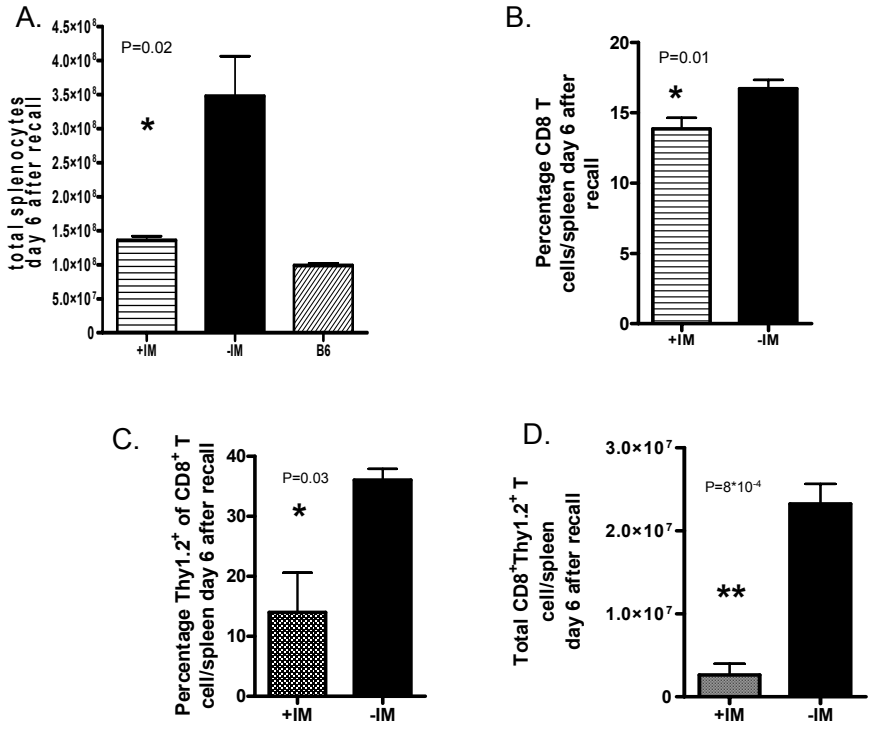

Figure 18. Total splenocyte counts, percentage $CD8^+$ and OT-1 ($CD8^+Thy1.2^+$) T cells, and total OT-1 T cells in spleen on day 7 after re-challenge of memory response. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with and without daily intraperitoneal injections of 75 mg/kg IM twice daily. The animals received a secondary challenge 1 LD_{50} of LM-OVA on day 29, with or without daily injections of IM, and sacrificed on day 35, and analyzed by flow cytometry. A. Total splenocytes on day 6 after recall. B. Percentage $CD8^+$ T cells in spleen on day 6 after recall. C. Percentage $CD8^+Thy1.2^+$ T cells in spleen on day 6 after recall. D. Total $CD8^+Thy1.2^+$ T cells in spleen on day 6 after recall. The data are representative of 3 independent experiments with 5 mice / group. Bars with * and ** represent $p < 0.05$ and $p < 0.001$ respectively.

Effect of IM on the secondary response of OT-1 T cells *in vivo*

Since IM decreased the IL-7Rα expression on OT-1 cells during the effector phase of the primary response, we wished to determine whether continuous IM administration would affect the secondary response. B6.Thy1.1 recipients received 10^6 LN OT-1 T cells and 0.1 LD_{50} of LM-OVA. On day 28, before re-challenge, we observed that the percentage of OT-1 memory cells (Figure 17A) in PBLs from mice that received IM was 40% less than in controls (Figure 17B). On the next day, the animals were re-challenged with 1 LD_{50} of LM-OVA, and the CD8 T cell response was assessed 6 days later. On day 6 after recall, the total splenocyte numbers decreased ~2.5 fold in IM-treated animals in relation to untreated controls (Figure 18A), while the percentage of total CD8 T cells in spleen decreased from ~18% in the untreated group to ~14% in the IM treated group (Figure 18B). In the same experiment, the percentage of OT-1 cells decreased from ~36% in controls to ~14% in IM treated mice (Figure18C). Further, the total number of OT-1 cells decreased ~4 fold in spleen (Figure18D). To assess the OT-1 cells in PBL, we gated on $CD8^+Thy1.2^+$ T cells in PBL (Figure 19A). In PBL, the mice that received IM had 60% fewer OT-1 cells (Figure 19B). These results indicate that in addition to a reduction in spleen size, the total percentages of CD8 cells, and the numbers of OT-1 cells, IM lowers the OT-1 memory response.

To determine the functional status of IM-treated memory OT-1 T cells, we cultured the spleen cells from the infected mice on day 6 after recall for 16 hours with SIINFEKL, and measured IFN-γ by ICS. Even though IM-treated animals had fewer

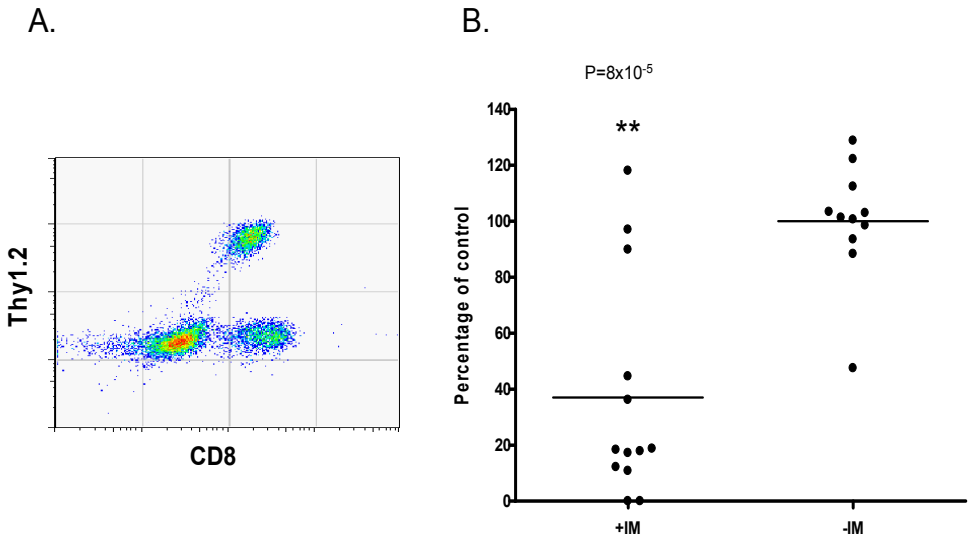

Figure 19. Effect of IM on OT-1 memory cells after re-challenge. Animals received OT-1 T cells and were infected with LM-OVA, with or without twice daily injections of IM. On day 29 they were re-challenged with LM-OVA, with or without daily injections of IM, and sacrificed on day 35. PBL were analyzed for the percentage of donor OT-1 T cells and normalized as in Figure 11. A. The dot plot is representative of memory $CD8^{+}Thy1.2^{+}$ cells after recall. B. Percentage of control response after recall. The percentage of OT-1 T of CD8 T cells in control animals averaged 43%. Data are from 4 independent experiments. Bars with ** represent $p < 0.001$.

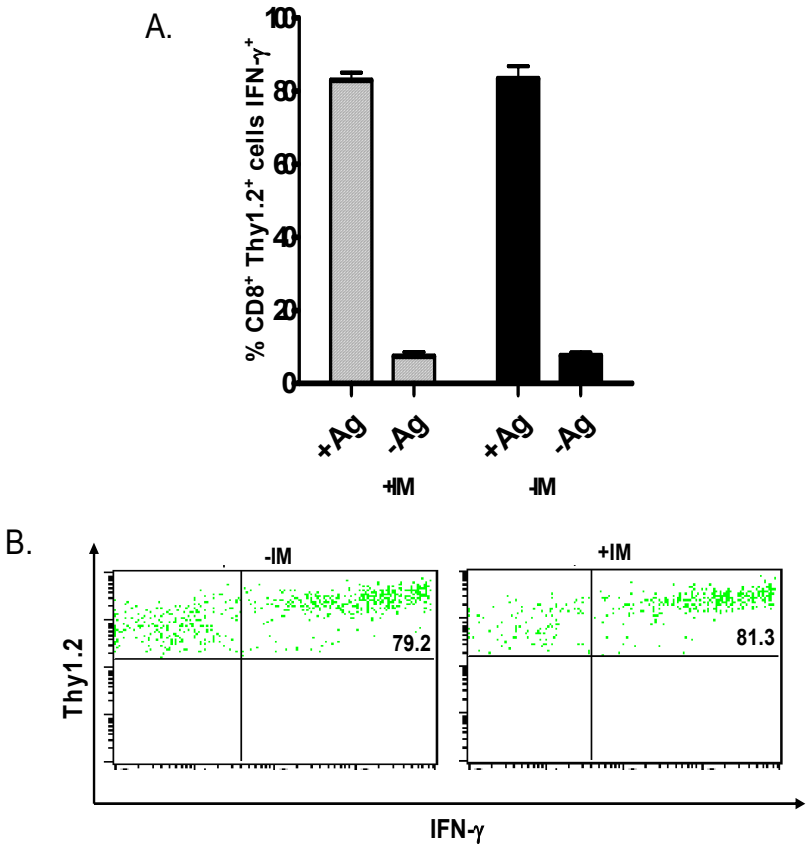

Figure 20. Effect of IM on the memory OT-1 cell response *in vivo* as determined by ex-vivo IFN-γ staining. Splenocytes from animals 6 days after re-challenge with LM-OVA were cultured overnight +/- cognate antigen (SIINFEKL) and +/- IM, and 16 hours later were stained for intracellular IFN-γ. A. Percentage $CD8^+Thy1.2^+$ $IFN\text{-}\gamma^+$ cells. B. The dot plots, gated on $CD8^+Thy1.2^+$, are representative data of $IFN\text{-}\gamma^+$ cells. Data are representative of 3 independent experiments with 4 animals in each group. The differences between the -IM and +IM groups are NS.

responding cells, no difference in IFN-γ^+ cells was detected between animals receiving vehicle versus IM (Figure 20A and 20B).

Effect of length of IM exposure on the memory OT-1 T cell response

Studies indicate that a naïve CD8 T cell is programmed to go through three phases during an immune response: differentiation, contraction and memory (Kaech et al., 2002b). Further, factors such as cytokine environment, costimulation, TCR avidity and epitope abundance can influence the CD8 T cell differentiation and development into a memory cell (Kaech et al., 2002b). Since continuous administration of IM lowered the OT-1 memory response, we tested whether administration of IM during priming, after priming, or prior to rechallenge differentially affected the OT-1 response. B6.Thy1.1 recipients received 10^6 LN OT-1 T cells and 0.1 LD_{50} of LM-OVA. On day 28, the animals were re-challenged with 1 LD_{50} of LM-OVA, and the CD8 T cell response was assessed 6 days later. To assess the duration of IM delivery on the memory recall CD8 T cell response, drug delivery either begun later and/or was withdrawn (WD) early. No consistent result was observed (Figure 21A). To determine if the duration of IM exposure influenced the extent of inhibition of memory cell responsiveness, we plotted the recall response as a function of days receiving IM. For example "WD day 7" and "begin at re-challenge" groups both received IM for only 7 days, whereas "WD at re-challenge" and "begin day 8" groups both received IM for 28 days. "Begin day 8, WD at re-challenge" group received IM for 21 days, while "+IM" group received IM for 35 days. The number of days that animals received IM was somewhat related to the extent of inhibition of the response since animals receiving IM during the full 35 days of the

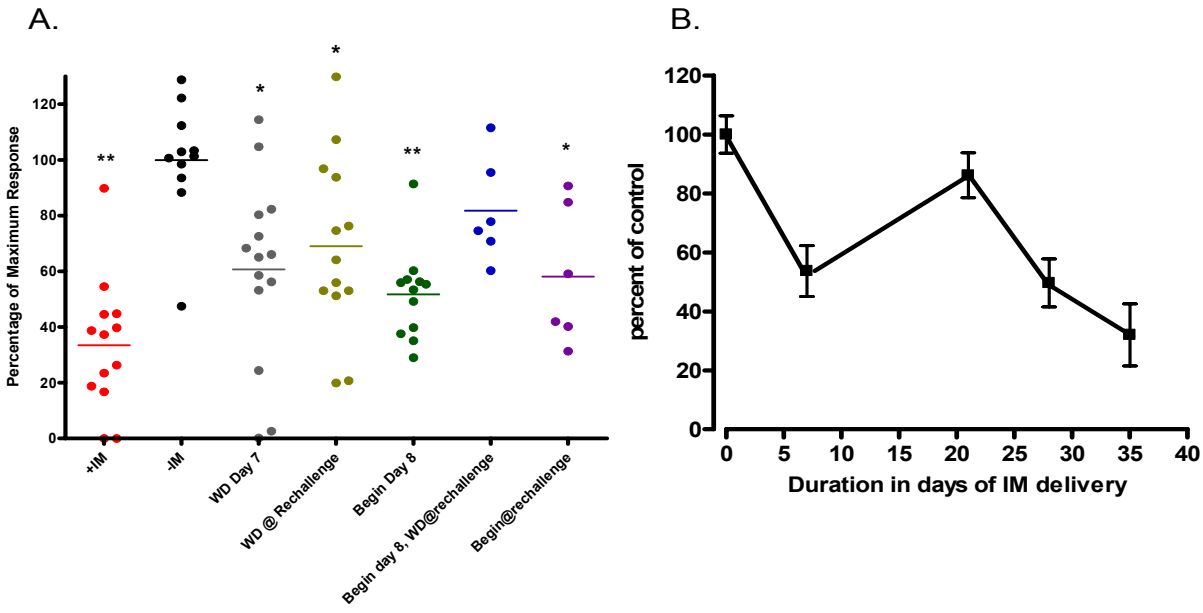

Figure 21. Effect of duration of IM exposure on the OT-1 recall response. Animals with primary and secondary infections of LM-OVA received daily injections of IM for the indicated intervals. (A) Percentage of maximum control response as assessed on day 6 after re-challenge. Termination of IM delivery is designated as "WD" (withdrawn). A late start of IM delivery is designated as "begin". The mice were divided into different groups consisting of 5 mice/ group. Group 1 received IM throughout the experiment, while group 2 received no IM throughout the entire experiment. Group 3 received IM during the first 7 days (primary response-WD day 7), and group 4 received IM up to day 28 (the day of re-challenge- WD @ re-challenge). Group 5 received IM after the first 7 days (after the primary response- begin day 8). Group 6 received IM after the first 7 days, and IM injections were terminated before re-challenge on day 28 (begin day 8, WD @ re-challenge). Group 7 received IM after re-challenge on day 28 (begin @ re-challenge). (B) The same data were then replotted as animals receiving IM for a total of 7, 21, 28 or 35 days. PBL were analyzed and normalized as in Figure 11. Results are representative of 4 different experiments. Bars with * represent $p<0.05$, ** represent $p<0.001$.

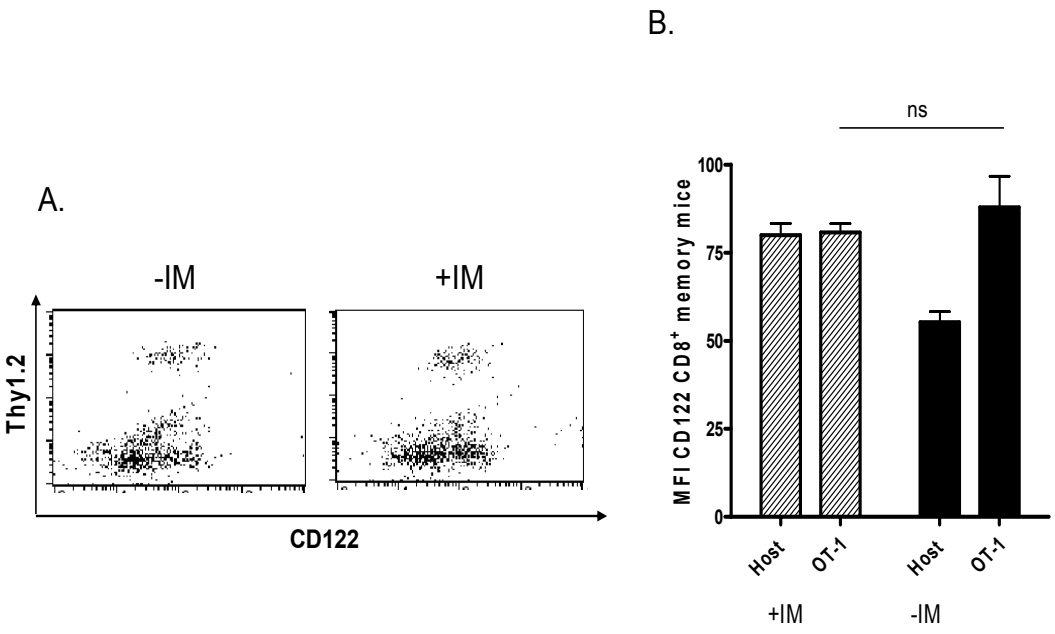

Figure 22. Effect of IM on expression of CD122 on OT-1 cells in memory response. Animals received OT-1 T cells and were infected with LM-OVA with or without twice daily injections of IM. On day 29 they were re-challenged with LM-OVA, with or without daily injections of IM, and sacrificed on day 35. The splenocytes were analyzed for $CD122^+$ by flow cytometry. A. The dot plots, gated on $CD8^+$, are representative of $CD122^+$ cells. B. Mean fluorescence intensity of CD122 on host $CD8^+$ and OT-1 T cells. The results are representative from 2 independent experiments with 4 mice in each group. The differences are NS.

experiment displayed greater inhibition of memory cell expansion after recall than those that received drug for shorter intervals (Figure 21B). This experiment did not answer whether the time of addition of IM determines which phase of CD8 T cell programming IM acts on.

Effect of IM on expression of CD122 and CD94 markers

To characterize the memory phenotype on IM-treated antigen-specific cells, we examined CD122 and CD94, markers characteristic of memory cells. It has been reported that memory CD8 T cells require contact with cytokines, particularly IL-15, for their survival (Berard et al., 2003). CD122 is the β chain for IL-15R, as well as that of IL-2R. Therefore, CD122 expression is high on memory CD8 T cells. The MFI of CD122 on antigen-specific cells remained unaltered between IM-treated and untreated groups (Figure 22A and 22B).

Expression of CD94/NKG2 on CD8 T cells is significantly increased upon infection with LM and other infectious agents. Therefore, CD94 could be a common marker for CD8 T cell activation (Moser and Loetscher, 2001). $CD94^{high}$ CD8 T cells display less apoptosis than $CD94^{int}$ cells (Gunturi et al., 2003). The expression of CD94 on OT-1 T cells, as measured by MFI levels, is unchanged on IM vs control treated animals (Figure 23A and 23B).

Effect of IM on expression of IL-7Rα on OT-1 memory cells

Expression of IL-7Rα has been linked to the generation of functional memory cells (Kaech et al., 2003). Since the expression of IL-7Rα was lower on OT-1 effector cells during the primary response, we tested whether IM alters IL-7Rα expression on

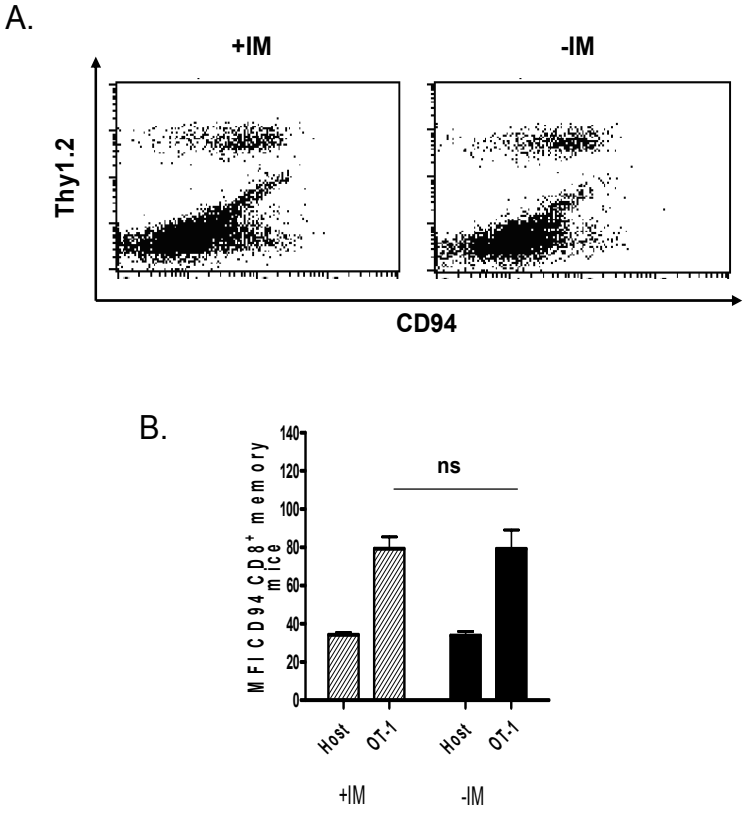

Figure 23. Effect of IM on expression of CD94$^+$ on OT-1 cells in memory response. Animals received OT-1 T cells and were infected with LM-OVA with or without twice daily injections of IM. On day 29 they were re-challenged with LM-OVA, with or without daily injections of IM, and sacrificed on day 35. The splenocytes were analyzed for CD94$^+$ cells by flow cytometry. A. The dot plots, gated on CD8$^+$, are representative of CD94$^+$ cells. B. Mean fluorescence intensity of CD94$^+$ on host CD8$^+$ and OT-1 cells. The results are representative from 2 independent experiments with 4 mice / group. The difference is NS.

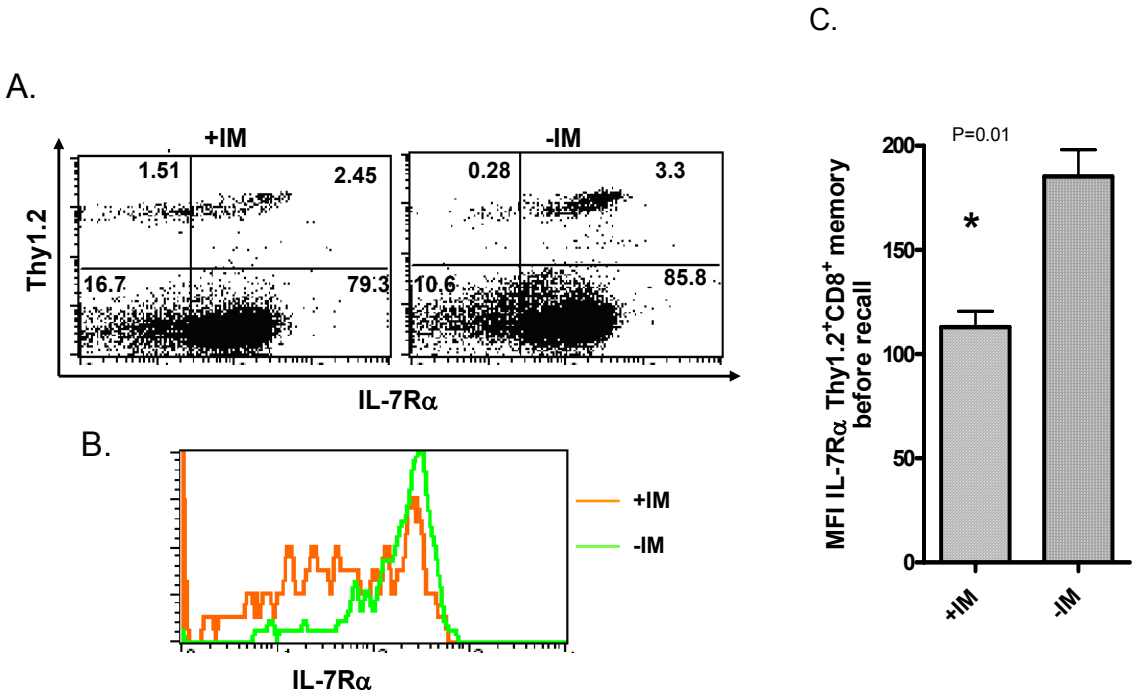

Figure 24. Effect of IM on expression of IL-7Rα$^+$ on OT-1 cells in memory before recall. Animals received OT-1 T cells and were infected with LM-OVA with or without twice daily injections of IM. On day 29, the blood was analyzed for IL-7Rα$^+$ (CD127$^+$) by flow cytometry. A. The dot plots, gated on CD8$^+$, are representative of IL-7Rα$^+$ on host and OT-1 CD8 T cells. B. Representative IL-7Rα histogram is gated on CD8$^+$Thy1.2$^+$ (OT-1) cells. C. The bar graph represents the mean fluorescence intensity (MFI) of OT-1 cells before recall in +IM treated and control mice. The results are representative from 3 independent experiments with 4 mice in each group. Bars with * represent $p<0.05$.

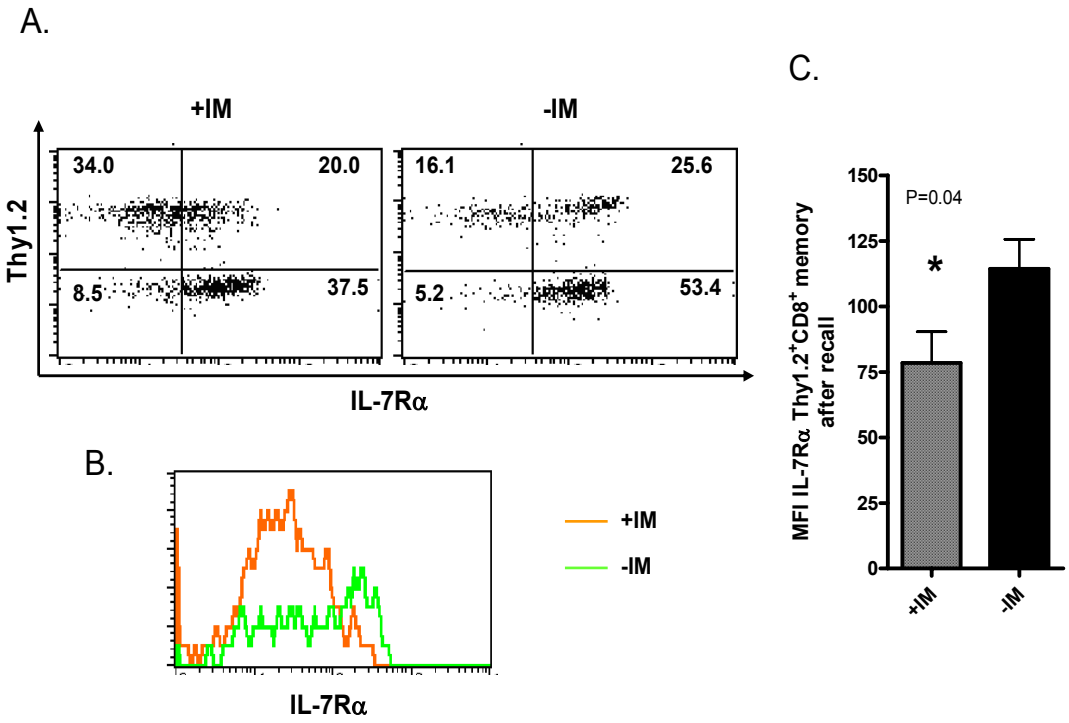

Figure 25. Effect of IM IL-7Rα$^+$ OT-1 cells in memory after recall. Animals received OT-1 T cells and were infected with LM-OVA with or without twice daily injections of IM. On day 29, they were re-challenged with LM-OVA, with or without daily injections of IM, and sacrificed on day 35. The blood was analyzed for IL-7Rα$^+$ (CD127$^+$) by flow cytometry. A. The dot plots, gated on CD8$^+$, are representative of IL-7Rα$^+$ on host and OT-1 CD8 T cells. B. Representative IL-7Rα histogram is gated on CD8$^+$Thy1.2$^+$ (OT-1) cells. C. The bar graph represents the MFI after recall on OT-1 cells in +IM treated and control mice. The results are representative from 3 independent experiments with 4 mice in each group. Bars with * represent $p<0.05$.

memory OT-1 cells before and after recall. Similar to the primary response, a positive IL-7Rα gate was set according to IL-7Rα staining of CD8 T cells on naïve untreated controls. IM decreased the expression of IL-7Rα on OT-1 T cells (Figure 24A). The ratio of IL-7Rα^{+}/ IL-7Rα^{-} cells of the OT-1 gate had a 7.4 fold reduction from the control to the IM treated group (Figure 24A). This IL-7Rα^{+}/ IL-7Rα^{-} ratio on host CD8 cells was only 2 fold less in IM treated animals as compared to controls. The histograms of IL-7Rα expression on OT-1 cells also showed a downregulation of IL-7Rα expression in the treated mice (Figure 24B). In addition, the MFI of IL-7Rα on OT-1 cells of IM treated animals was also decreased before recall (Figure 24C). After recall, the ratio of IL-7Rα^{+}/ IL-7Rα^{-} cells of in the OT-1 gate had a 2.7 fold reduction from the control to the IM treated group (Figure 25A). The histograms of IL-7Rα also showed a downregulation of IL-7Rα expression on OT-1 cells in IM-treated animals (Figure 25B). Further, the MFI of IL-7Rα also decreased after recall (Figure 25C). In additional analysis, the IL-7Rα expressing cells were gated for expression of high levels of IL-7Rα (IL-7Rα^{hi}) as in the primary response. Before recall, the percentage of IL-7Rα^{hi} cells was lower on memory cells in IM-treated mice compared to untreated controls. The percentage of IL-7Rα^{hi} expressing cells decreased from 86% (range 78-88%) to 54% (range 46-55%) (Figure 26A). Furthermore, after recall IM treatment also resulted in a lower percentage of IL-7Rα^{hi} memory OT-1 T cells compared to untreated controls. The percentage of IL-7Rα^{hi} cells in IM-treated mice was 9% (range 8-15%) compared to 34%

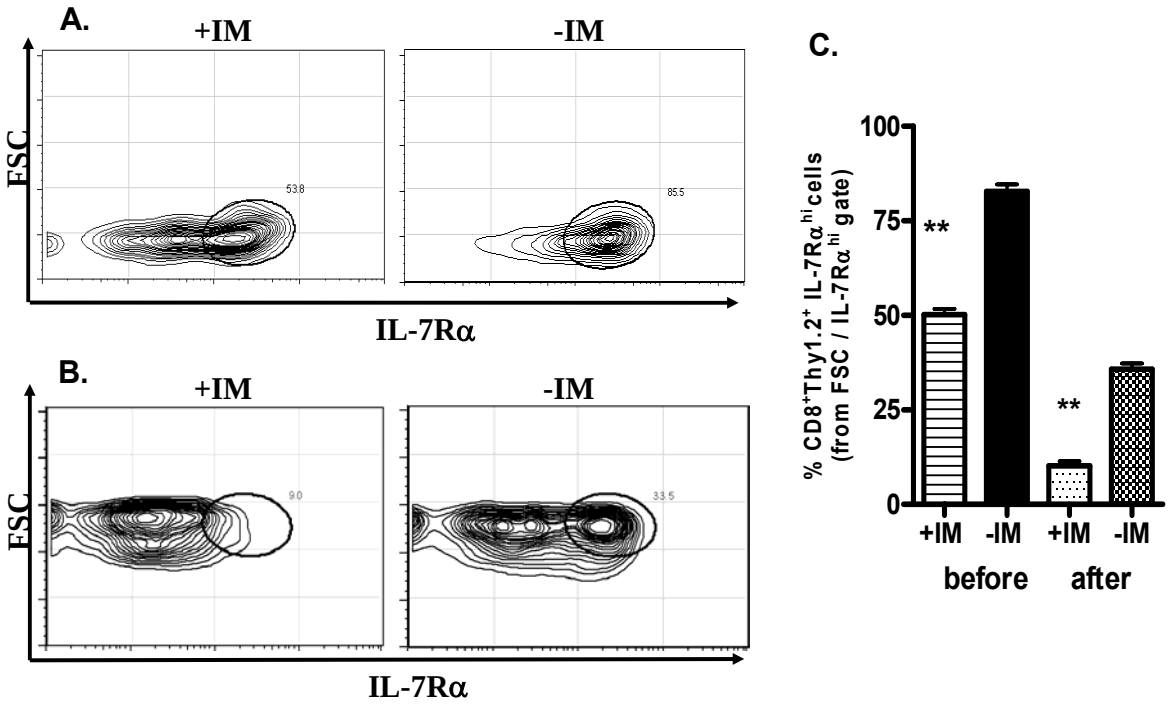

Figure 26. Effect of IM on IL-7Rα expression on OT-1 cells. Spleen cells were gated on $CD8^{+}Thy1.2^{+}$ OT-1 T cells. (A) IL-7Rα expression on PBL OT-1 memory cells before recall response (day 28). In this experiment, the percentage CD8 T cells in PBL averaged 19% and percentage OT-1 of CD8 T cells averaged 5% in control animals. The percentage of IL-$7R\alpha^{hi}$ expressing cells ranged from 78-88% in controls (mean 82.8± 4.0) and from 46-55% in IM-treated animals (mean 50.2± 3.4), ($p < 0.001$). (B) IL-7Rα expression on spleen OT-1 memory cells 6 days after secondary infection (day 35). In this experiment, the percentage of CD8 T cells averaged 31% and percentage of OT-1 T cells of CD8 T cells averaged 46% in control animals. The percentage of IL-$7R\alpha^{hi}$ expressing cells ranged from 32-40% in controls (mean 35.8± 3.4) and from 8-15% in IM-treated animals (mean 10.2± 2.9), ($p < 0.001$). (C) The percentage of IL-$7R\alpha^{hi}$ expressing cells (as gated in A and B) in controls and IM-treated animals. Results are representative of 3 independent experiments with 5 animals in each group.

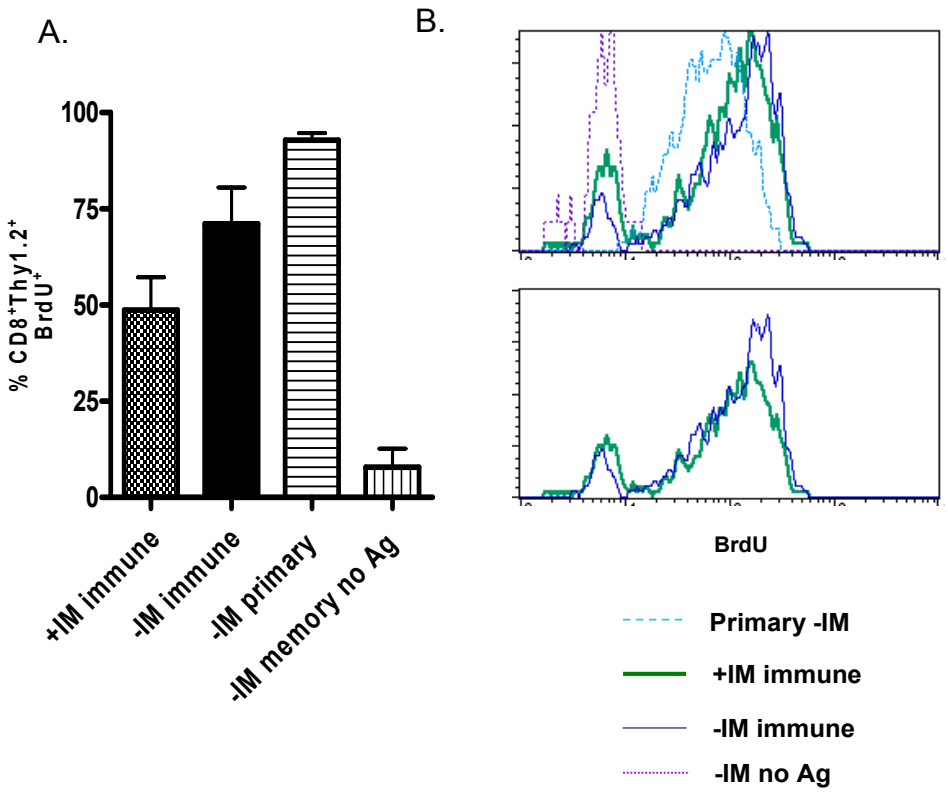

Figure 27. ***In vivo*** **expansion of OT-1 memory cells.** B6.Thy1.1 animals received OT-1 T cells and were infected with LM-OVA. On day 29, the groups were fed BrdU in their drinking water and challenged with LM-OVA. On day 35 the OT-1 T cells were analyzed for BrdU accumulation. Controls include untreated memory mice that did not receive LM-OVA re-challenge, and untreated mice that received LM-OVA during a primary challenge. A. Percentage $CD8^+Thy1.2^+$ $BrdU^+$ cells. B. The histograms are representative for BrdU accumulation in each group. The top histogram includes all the groups, while the bottom histogram only includes the -/+IM immune groups, from the same experiment. The difference between IM-treated mice versus untreated controls is NS. Results are representative of 2 independent experiments with 5 animals in each group.

(range 32-40%) in untreated controls (Figure 26B). When the percentage of IL-7Rα^{hi} OT-1 cells from the FSC/ IL-7Rα dot plots were plotted, a reduction was observed in the IM-treated groups (Figure 26C). Therefore, not only did IM decrease the number of memory cells and their response to antigen, but it also decreased the percentage of cells that express high levels of IL-7Rα.

Effect of IM on the expansion of memory OT-1 cells

During the primary response, treatment of either OT-1 T cells or the host cells with IM did not influence *in vivo* proliferation of OT-1 cells. This was consistent because IM did not influence the primary response of OT-1 cells. Since there were fewer OT-1 cells in IM-treated mice after re-challenge with LM-OVA, we determined whether IM influences the proliferation of OT-1 memory cells *in vivo*. Mice received BrdU continuously during re-challenge with LM-OVA. 50% of the cells in IM-treated mice incorporated BrdU as compared to 72% of cells in untreated mice (Figure 27A). The BrdU accumulation histograms also show a slight decrease in proliferation (Figure 27B). However, this difference was not statistically significant.

Ability of IM-treated mice to clear LM infection during the recall response

Since IM treatment reduced the CD8 memory OT-1 response to LM-OVA, we determined whether IM altered the function of these memory cells. This was done by testing the ability of immune versus naïve animals to clear a lethal dose of LM-OVA. Recipients received 50 LD_{50} of LM-OVA at the time of recall. Since clearance of LM from immune mice is almost complete on day 2, spleens and livers were analyzed on day

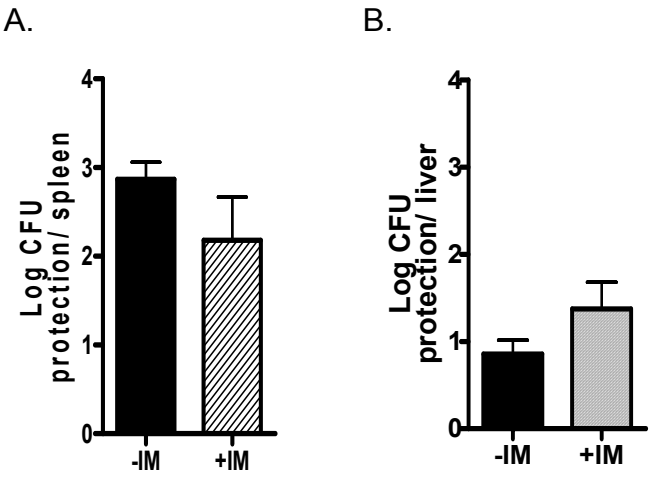

Figure 28. ***In vivo*** **function of OT-1 memory cells.** C57BL/6 mice were infected with 0.1 LD_{50} LM-OVA on day 0, and re-challenged with 50 LD_{50} LM-OVA on day 29. On day 30, the spleens and livers were homogenized and LM-OVA CFUs were determined. Controls include naïve animals that received 50 LD_{50} LM-OVA. Data shows log CFU protection in immune versus naïve animals in (A) spleen, and (B) liver. Log CFU protection was calculated by subtracting the average log bacterial burden in the spleens and livers of immune LM-OVA mice from the average log bacterial burden in the same organs of LM-OVA naïve mice. Differences are NS. Results are representative of 3 independent experiments with 4 animals in each group.

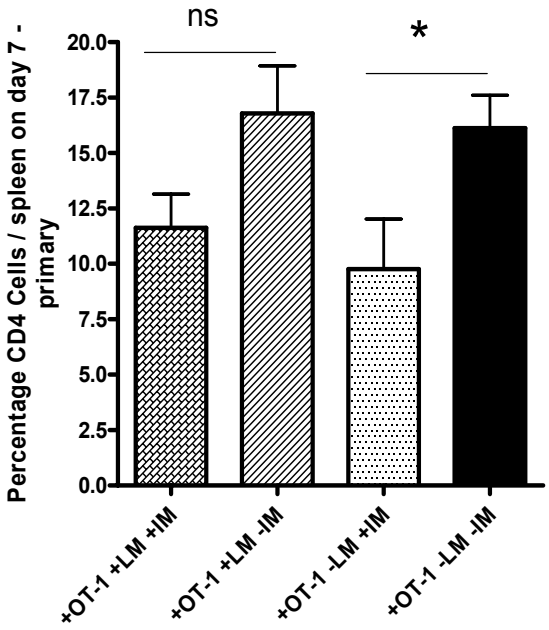

Figure 29. Percentage of CD4 T cells in spleen on day 7. B6.Thy1.1 mice received 10^6 OT-1 cells (+OT-1) and 0.1 LD_{50} LM-OVA (+LM) along with daily intraperitoneal injections of 75 mg/kg IM (+IM) twice daily. On day 7, the animals were analyzed for CD4 T cells by flow cytometry. Control animals did not receive LM-OVA infection (-LM), or did not receive IM (-IM). Results are representative of one experiment with 6 mice / group. Bars with * represent $p<0.05$.

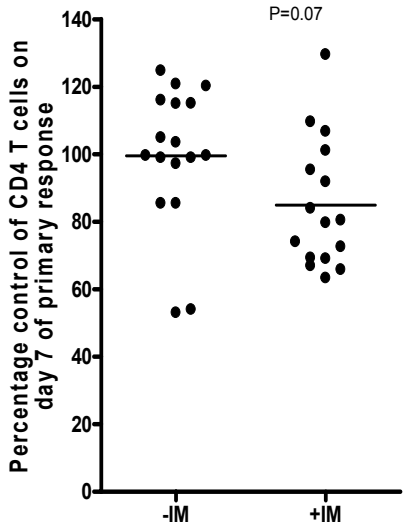

Figure 30. Percentage control of CD4 T cells in blood on day 7. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with daily intraperitoneal injections of 75 mg/kg IM twice daily. On day 7, PBL were analyzed for the percentage of CD4 T and normalized as in Figure 11. Results are from 4 independent experiments with 4-5 mice / group. Differences are NS.

one to maximize the chance to see an effect from IM (Seaman et al., 2000). Our results reveal that IM did not change the ability of memory T cells in these animals to clear LM (Figure 28). In the spleen, the log CFU protection in IM-treated mice was 2.2 fold similar to the 2.8 fold in the IM untreated group (Figure 28A), while in liver the log CFU protection in IM-treated mice was 1.3 fold similar to the 0.9 fold in the IM untreated group (Figure 28B).

Effect of IM on CD4 T cells *in vivo*

CD4 T cell help for generation of a primary anti-LM CD8 T cell response is not necessary, while its presence is important for the generation of a fully functional anti-LM CD8 T cell response (Sun and Bevan, 2003; Shedlock and Shen, 2003). Because we observed a lower OT-1 memory response, we wondered if this was due to a defective CD4 T cell help. We investigated whether the CD4 T cells in our system were affected by administration of IM. B6.Thy1.1 recipients received 10^6 LN OT-1 T cells and 0.1 LD_{50} LM-OVA with or without daily injections of 75 mg/kg IM. On day 7, the percentage of CD4 T cells in the spleen did not have a significant change between the IM-treated and untreated groups in presence of an LM infection (Figure 29). While in the absence of an LM infection, IM-treated hosts displayed a lower CD4 T cell percentage (Figure 29). Consistent with this, the percentage control of CD4 T cells during the primary response did not change (Figure 30).

It has been shown that in a LM infection, the absence of CD4 T cell help shortly after the expansion phase (day 7-8) compromised the generation of fully functional CD8 memory response cells (Huster et al., 2004; Sun et al., 2004). On day 28, the percentage

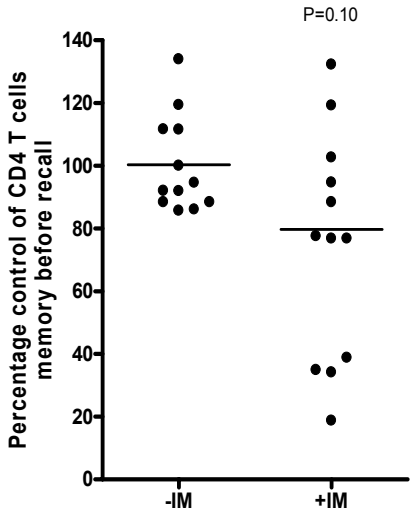

Figure 31. Percentage control of CD4 T cells in blood before recall. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with daily intraperitoneal injections of 75 mg/kg IM twice daily. On day 28, PBL were analyzed for the percentage of CD4 T and normalized as in Figure 11. Results are from 3 independent experiments with 4 mice in each group. Differences are NS.

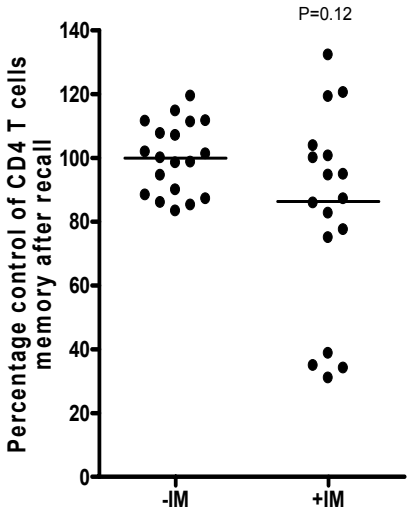

Figure 32. Percentage control of CD4 T cells in blood after recall. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with daily intraperitoneal injections of 75 mg/kg IM twice daily. On day 29, the animals were re-challenged with LM-OVA, with or without daily injections of IM, and sacrificed on day 35. PBL were analyzed for the percentage of CD4 T cells and normalized as in Figure 11. Results are from 4 independent experiments with 4-5mice / group. Differences are NS.

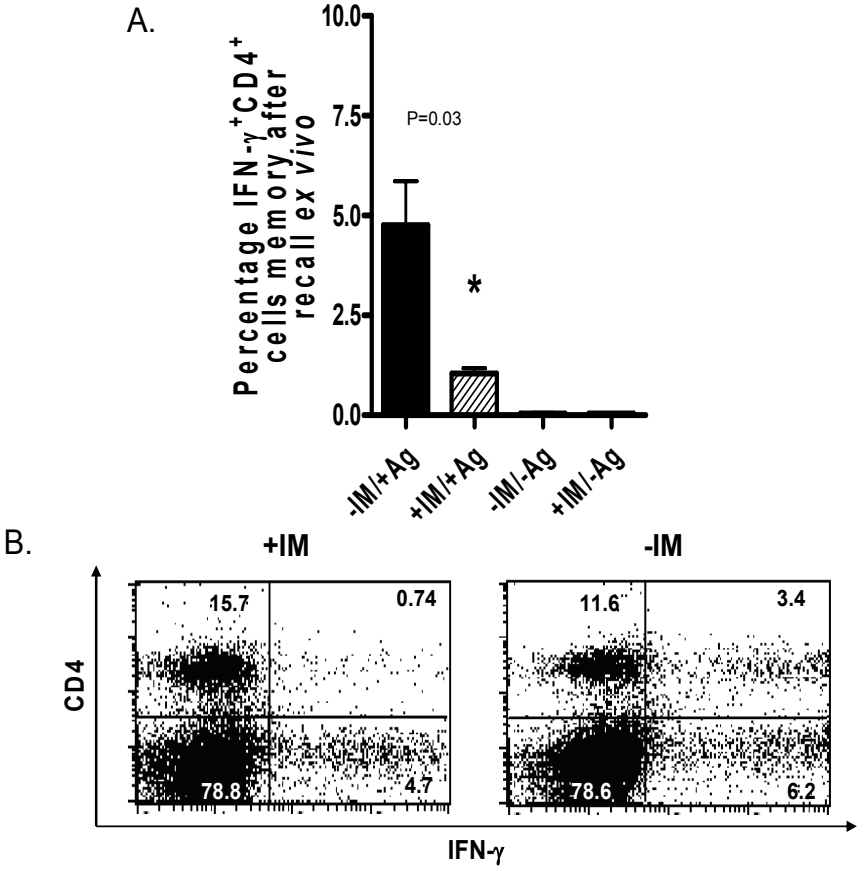

Figure 33. Percentage of IFN-γ^+ CD4 T cells in spleen after recall. B6.Thy1.1 mice received 10^6 OT-1 cells and 0.1 LD_{50} LM-OVA along with daily intraperitoneal injections of 75 mg/kg IM twice daily. On day 29, the animals were re-challenged with LM-OVA, with or without daily injections of IM, and sacrificed on day 35. Splenocytes were cultured overnight +/- α-CD3 and +/- IM, and 16 hours later were stained for intracellular IFN-γ^+ $CD4^+$ T cells. A. Percentage of IFN-γ^+ $CD4^+$ T cells. The percentage of $CD4^+$ T cells in this experiment ranged from 13.2-16.7% in IM-treated animals and 11.5-17.1% in controls. The percentage of IFN-γ^+ $CD4^+$ T cells ranged from 0.7-1.4% in IM-treated animals (mean 1.0± 0.3) and from 2.1-8.6% in controls (mean 4.7± 2.4), ($p < 0.05$). B. The representative CD4 / IFN-γ dot plots (gated on $CSFE^+$ cells). Data are representative of 3 independent experiments with 5 animals / group. Bars with * represent $p<0.05$.

control of CD4 T cells during memory before recall did not alter between the IM-treated and control group (Figure 31). Further, the animals were re-challenged on day 28, and CD4 T cell percentages were assessed on day 7 after recall. The percentage control of CD4 OT-1 cells during memory after recall remained unaltered between the IM-treated and control group (Figure 32). To determine the functional status of IM-treated memory CD4 T cells, we cultured the spleen cells from the infected mice on day 6 after recall for 16 hours with anti-CD3, and measured IFN-γ by ICS. Even though IM-treated animals had similar CD4 T cell percentages during the memory response, the percentage of IFN-γ positive CD4 cells after recall was reduced from 6.5% in untreated controls to 1.2% in IM-treated animals (Figure 33A). A similar 5-6 fold reduction in the percentages of $CD4^+$ IFN-γ^+ is also observed on the dot plots (Figure 33B). Our results indicate that in this system while IM does not affect the CD4 percentages during the primary or secondary responses quantitatively, it depresses the CD4 T cells during the memory response quantitatively as measured by production of IFN–γ.

Chapter Five

Discussion

The Effect of IM *in vitro* on primary and memory CD8 T cells

Studies show that IM has been shown to suppress *in vitro* T cell responses, along with its influence on CML cells. Exposure to IM leads to a stop in proliferation, and to an induction of apoptotic cell death in ph^+ cell lines derived from CML patients (Druker et al., 1996; Deininger et al., 1997). Further, these inhibitory effects are also observed on hematopoietic cells derived from fresh leukemic cells of CML patients (Druker et al., 2002). The inhibitory effects on hematopoietic cells occur at a higher IC_{50} value (1μM) than the IC_{50} for ph^+ cells (0.5μM). Investigations on the effects of IM *in vitro* on T cells derived from normal human peripheral blood report that IM inhibits T cell proliferation and IFN-γ production, while it induces activation induced cell death (Cwynarski et al., 2004; Seggewiss et al., 2005; Dietz et al., 2004; Boissel et al., 2006b). These effects are seen in the presence of stimulation through TCR and CD28, or with mitogens or antigen. However, the effect of IM on the fate of activating markers CD25 and CD69 on PBL derived T cells and T cell lines remains unresolved (Seggewiss et al., 2005; Dietz et al., 2004). While the effects of IM on primary T cells has been investigated *in vitro*, the studies investigating the effects of IM on primed T cells are few (Seggewiss et al., 2005). In that, we investigated the effect of IM on primary and memory T cell responses *in vitro*, and *in vivo* in an adoptive transfer model utilizing the LM bacteria as a pathogen.

In our studies, addition of IM to *in vitro* cultures of splenocytes inhibited primary and secondary T cell responses in a dose-dependent manner. This inhibition decreased proliferation and IFN-γ production, and increased apoptosis induced by activation induced cell death (AICD). However, the effects of IM were not detected unless IM concentrations were $\geq 7.5\mu M$. At 5μM no inhibition was detected. Our results are similar to others showing inhibitory effects of IM on human PBLs (Cwynarski et al., 2004; Seggewiss et al., 2005; Dietz et al., 2004; Zipfel et al., 2004). However, these studies observe the dose dependent effect at 1μM concentrations, whereas we observed the dose dependent effect at $>$ 5μM concentrations. The slightly higher dose required for inhibition that we report here could be related to the greater sensitivity to IM of human versus mouse cells. We also tested the sensitivity of primed cells to IM. We observed an inhibition of proliferation and IFN-γ production, along with induction of activation induced cell death. Our *in vitro* results are similar to data showing inhibition of the response of human PBLs to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) by IM although we only noted this at concentrations of $\geq 7.5\mu M$ (Seggewiss et al., 2005).

Effective Dosage and Plasma concentrations of IM in C57BL/6 animals

For *in vivo* studies, plasma concentrations and dosage of IM need to be similar to those used in therapeutic studies of CML. Studies in the bone marrow transduction/transplantation model of CML use a range of 50mg/kg to 150 mg/kg of IM in treatment of CML bearing BALB/C mice (Wolff and Ilaria, Jr., 2001; Druker et al., 1996). Early pharmacokinetic studies at Novartis showed that in mice, IM is rapidly

absorbed and reaches pharmacologically relevant concentrations in the plasma, with a $t_{1/2}$ of approximately 1.3 hours (Druker, 2002). To determine the effective dose of IM on tumor growth *in vivo*, BCR-ABL transformed 32D cells were injected into syngeneic mice (Druker et al., 1996). Doses of 2.5 to 50 mg/kg of IM, started one week after cell injection and delivered on a once daily schedule, caused dose-dependent inhibition of tumor growth. Similar experiments in nude mice using KU812 cells, a BCR-ABL$^+$ human cell line, showed the need for continuous inhibition of BCR-ABL kinase activity to achieve maximal antitumor effects (le et al., 1999).

In the following experiments, in order to assure continuous blockage of $p210^{BCR\text{-}ABL}$ tyrosine kinase, the treatment schedule was optimized to 3 times daily administration of 50mg/kg IP for 11 consecutive days. This resulted in tumor-free survival of mice injected with KU812 BCR-ABL$^+$ cells (le et al., 1999). With the same schedule, established tumor nodules began to regress 48 hours after beginning of the treatment, and by day 8, no treated animal had measurable disease. In the murine transduction-transplantation model of CML (BMT), treatment schedule of 50mg/kg IM in the morning and 100 mg/kg in the evening in BABL/C mice prolonged survival (Wolff and Ilaria, Jr., 2001). Our studies on dose determination on C57BL/6 mice indicate that the B6 strain of mice tolerate IM at 75mg/kg, and not 150mg/kg. The differences we see in drug tolerability are likely due to strain differences between C57BL/6 and BALB/C.

Clinical trials studies use 400mg of IM as the standard dose for CML therapy. At 400mg, peak IM levels at steady state are ~ 4.6μM and trough levels are ~ 2.13μM (Peng et al., 2004b). Furthermore, IM exposure is dose proportional for a dose range of 25 to

1,000mg daily, indicating that higher doses of IM will result in higher μM concentrations in plasma (Peng et al., 2004b). Our experiments achieve plasma concentrations of 5.6 to 11μM with a mean 8.7 ± 2.7 at 2 hours. This is consistent with other published data where plasma levels of IM after 2 hours of IM delivery to mice were determined (Wolff et al., 2004). These plasma levels deliver therapeutic effects in a CML murine model (Wolff and Ilaria, Jr., 2001; Wolff et al., 2004). IM has a $t_{1/2}$ of approximately 1.3 hours in mice (Druker et al., 1996). This implies that our levels stay at a therapeutic range for CML for 4 hours.

Effect of IM on LM infection

To assess the effect of IM on an *in vivo* immune response, we chose to use the LM infection model. First, we had to assess whether IM has a direct effect on LM growth. There was a modest effect of IM on bacterial growth during the early stages of infection in that following injection of a low dose of LM-OVA, IM slightly decreased bacterial colonies in spleen but not liver. This effect was detected on day 1 but not on day 3. This suggests that IM increases the early inflammatory response to LM. This can be in that IM has positive immunomodulatory effects on DC (TipDC), if IM exposure time is short (Zeng et al., 2004; Sato et al., 2003). However, in the LM model, antigen dosage affects the initial expansion of CD8 T cells, but it does not influence the contraction, and the subsequent CD8 response (Badovinac et al., 2002). Therefore, the decrease in bacterial colonies in spleen we observed would not be expected to play a role in the subsequent adaptive response.

Effect of IM on the primary response

In vivo, IM did not influence the primary response of OT-1 CD8 T cells specific for the cognate ovalbumin epitope. This was demonstrated by our findings that the extent of *in vivo* expansion and IFN-γ production by cells undergoing a primary response was not affected by IM. In addition, the expression levels of activating markers CD122 and CD94 were unaltered. While IM did not affect the percentages of CD8 T cells, it decreased the total number of cells in the spleen on day 7. This indicates that the total numbers of host CD8 and OT-1 cells decreased during the primary response. Therefore, primary response as measured by total number of OT-1 cells decreased.

The only phenotypic alteration that we noted during the primary response is the decrease in expression of the memory marker, IL-7Rα. Untreated controls display a higher percentage of IL-7Rα^{hi} OT-1 cells. The relevance of IL-7Rα for this study will be discussed later. But, in that naïve cells need IL-7 for survival, the expression of IL-7Rα is high on naïve cells. Upon activation, the expression of IL-7Rα decreases. But about 5-10% of activated CD8 T cells early in response, as early as day 7, express very high levels of IL-7Rα (IL-7Rα^{hi}) (Huster et al., 2004; Kaech et al., 2003). These IL-7Rα^{hi} cells persist throughout the response and constitute the functional memory cells later in response. Our primary response results indicate that some cell population in the spleen is changing quantitatively, and that although the primary response to LM-OVA as measured by percentage of control response remains the same, the survival of OT-1 cells as traced by the memory marker IL-7Rα is maybe altered.

Effect of IM on the secondary response

When we assayed for memory OT-1 CD8 T cells, we observed that the percentage of CD8 antigen-specific cells decreased. Further, their response upon re-challenge with LM-OVA was lower. This correlated with a decrease in total OT-1 numbers in the spleen. Moreover, in IM-treated mice the total number of splenocytes and the percentage of CD8 T cells on day 7 after recall also decreased. This indicates that IM after a second re-challenge, not only decreases the antigen-specific CD8 T cell response, but it also decreases the host CD8 T cell percentages, and it could possibly have an effect on other cell types. Our data agree with previous data showing that IM inhibits the delayed type hypersensitivity (DTH) response, although the cell type(s) involved in the inhibition of this response was not described (Dietz et al., 2004).

It is possible that the decrease in an OT-1 memory response is due to a direct effect of IM on OT-1 cells. *In vitro*, studies that utilize activation by mitogens or through TCR, have shown that IM interferes with the TCR/ABL tyrosine kinase pathway (Zipfel et al., 2004; Dietz et al., 2004). This results in reduced levels of activated NF-κB transcription factor, and decreased phosphorylation on ZAP70, along with reduced signaling through LCK and Erk. It is possible that memory OT-1 cells would display a similar decreased signaling through the TCR. This can be assessed by Western blots of memory OT-1 cells probing for phosphorylated forms of ZAP70, LCK and Erk, or Northern blots of OT-1 cells probing for activated form of NF-kB. However, there are limitations. We can test a direct effect of IM treatment on OT-1 T cell TCR signaling *in vitro*. However, a test of a

direct effect on OT-1 cells that have received IM treatment *in vivo* would not exclude indirect contributions (through APC or CD4 cells) to a direct affect on TCR signaling.

Effect of IM on CD122

Decreased memory OT-1 responses could be due to altered responses to pro-survival cytokines, in particular IL-2 and IL-15. Both IL-2R and IL-15R belong to γc family of cytokines and therefore share the γc chain. In addition, IL-2R and IL-15R share their β chain, CD122 (Tsudo et al., 1986). Although these cytokines share their γ and β chains, their functions are different. This is demonstrated by the different phenotypes of mice deficient in IL-2 or IL-15. Mice that are deficient in IL-2 or IL-2R display a massive enlargement of peripheral lymphoid organs that is associated with polyclonal T and B cell expansion, and develop autoimmunity (Sadlack et al., 1994; Willerford et al., 1995). In contrast, IL-$15^{-/-}$ or IL-$15R\alpha^{-/-}$ mice only display a marked reduction in numbers of NK, NK-T and $CD8^{+}$ T memory cells (Kennedy et al., 2000; Lodolce et al., 1998). Consistent with these observations, IL-2 plays a crucial role in persistence of peripheral self tolerance, while IL-15 favors persistence of memory CD8 and CD4 T cells. Studies suggest the differential function of IL-2 and IL-15 is due to βγ chain signaling that is regulated by the concentration of the cytokine (Zambricki et al., 2005). This phenomenon, the regulation of cytokine signals by cytokine concentrations, is also demonstrated by the effect of IL-2 on regulatory CD4 cells. The constitutive expression of IL-2Rα (CD25) on regulatory $CD4^{+}$ cells, enables these cells to express the high-

affinity trimeric αβγ receptor and thereby use basal levels of IL-2 for survival (Malek and Bayer, 2004; Fontenot et al., 2005).

With relation to IL-2 involvement in CD8 memory cells, studies utilize T cell depleted IL-2Rα$^{-/-}$ and wild type bone marrow chimeras in irradiated B6 mice. IL-2 signals to antigen-specific CD8^{+} T cells have little effects on the number of developing effector and memory cells, but IL-2 signaling during priming is crucial for the expansion of a robust secondary response (Williams et al., 2006). With regards to IL-15 dependency of CD8 memory cells, there are two subsets as identified by expression of CD122. About 70% of CD8 memory cells display high levels of CD122. This CD122^{hi} population is highly dependent on IL-15 for IFN-induced bystander proliferation, as well as for turnover and survival (Judge et al., 2002). The remaining 30% of memory CD8^{+} cells are CD122^{lo} and IL-15 independent. This population is unaffected by IFN-induced bystander proliferation. Moreover, the CD122^{lo} population requires MHC I for proliferation, and closely resembles the antigen dependent memory CD8 T cells found in chronic viral infections (Judge et al., 2002; Boyman et al., 2006).

The expression of CD122 remains low on naïve cells, but is upregulated upon activation on effector cells, and memory cells that have received a re-challenge. Enhanced expression of CD122 on CD8 memory cells confers cellular responsiveness to IL-15 (Intlekofer et al., 2005). A lower level of IL-15 responsiveness, measured by lower CD122 levels in IM-treated animals, point to decreased survival of OT-1 memory cells. However, our data show the expression of CD122 on OT-1 cells, either in primary or secondary response, is not altered in IM-treated animals. This indicates the presence of

IM does not change the responsiveness of memory OT-1 cells to IL-15. Further studies could test the IL-2 responsiveness of IM-treated OT-1 cells during the priming period by co-staining these cells for the β and α chains of IL-2R (CD122 and CD25 respectively).

Effect of IM on CD94

Another potential explanation for a reduced memory OT-1 response addresses the role of CD94. CD94 is a C-type lectin that in association with either activating or inhibitory NKG2 receptors, confers activating or inhibitory signals. In mice, most CD94/NKG2 receptors on CD8 T cells are the inhibitory CD94/NKG2A heterodimers (Gunturi et al., 2003). Although few CD8 T cells from a naïve mouse express CD94/NKG2 receptors, 50% of CD8 T cells in a LM secondary response are $CD94^{hi}$ (Gunturi et al., 2003). A high expression of CD94 on CD8 T cells correlates with a lower level of apoptosis (Gunturi et al., 2003). CD94 expression is very low in naïve cells, but in effector cells its expression rises to very high. In memory cells that have been re-challenged, its expression is high. Our data show that both during the primary response and the memory response, there is not a change in CD94 expression on OT-1 cells. A lower level of CD94 expression on IM-treated animals could lend further evidence to apoptosis serving as a mechanism of CD8 memory depression. Our results, however, indicate that CD94 expression on OT-1 memory cells is not an indicator of change during IM treatment.

It would be interesting to know if IM affects other hallmark memory markers. CD8 memory cells are divided into T_{CM} and T_{EM} groups. T_{CM} express high levels of CD62L, CCR7 and CD27, and are found in secondary lymphatic organs . T_{EM} express low levels

of CD62L, CCR7 and CD27, and are found primarily in the peripheral tissues. CD62L (L-selectin) molecule guides naïve and memory T cells to lymph nodes and Peyer's patches. CCR7 is a chemokine receptor that along with CD27, a costimulatory molecule, assists in migration of memory T cells to the lymph nodes. Therefore, co-staining with CD62L and CCR7 alongside with IL-7Rα on transferred memory cells would indicate if IM is affecting other memory markers.

Effect of IM on IL-7Rα

Another rationale for a lower OT-1 response is an altered response to IL-7. IL-7 is a cytokine involved in homeostatic proliferation of CD8 T cells in lymphopenic animals (Goldrath et al., 2002). It is also needed for the survival of both naïve and memory T cells (Tan et al., 2001; Ku et al., 2000; Surh et al., 2002; Surh and Sprent, 2005). IL-7R is a member of γc chain cytokine receptor family, and is composed of the high affinity IL-7Rα in complex with γc chains. More recent data indicate that IL-7Rα expression is required for effective memory cell generation, and effector CD8 T cells that retain high levels of this receptor give rise to long-lived memory cells (Kaech et al., 2003; Huster et al., 2004). Further, expression of IL-7Rα^{hi} early in the response correlates with the functional quality of memory cells generated, and their adoptive transfer leads to protective immunity (Huster et al., 2004; Fuller et al., 2005; van Leeuwen et al., 2005). The decrease in IL-7Rα^{hi} expression on primary and memory OT-1 cells exposed to IM is consistent with their impaired secondary response. Furthermore, this data agrees with other published data on memory responses in LM infected animals where an IL-7Rα^{hi}

population early in response correlated with a functional memory response (Huster et al., 2004).

Studies suggest that T_{EM} differentiate into T_{CM}, and T_{CM} serve as a reservoir for generating a large pool of secondary effectors (Kaech et al., 2003; Wherry et al., 2003; Kaech et al., 2002a). Further, lineage studies in the LM model confirm the unidirectional development of T_{CM} into T_{EM} (Huster et al., 2006). In that we are analyzing the spleen and blood, both T_{CM} and T_{EM} are present. The memory staining of IL-7Rα along with CCR7 and CD62L delineates which subset of T_M cells is affected. Whatever the results of T_M subset identification are, the bacterial clearance assay of splenocytes did not show a functional impairment . The log CFU protection counts in the LM clearance assay of memory OT-1 cells indicate that both control and IM-treated mice clear a lethal dose of bacteria. Moreover, it would be interesting to know if these IM treated IL-7Rα cells would proliferate or function differently in a secondary host. A secondary adoptive transfer of sorted memory IL-7Rα^{hi} and IL-7Rα^{lo} OT-1 cells followed by an LM-OVA infection would delineate if proliferative or functional capacities of the two groups are different. Similar studies using the LCMV and LM models have used adoptive transfer of sorted IL-7Rα^{hi} and IL-7Rα^{lo} cells (Huster et al., 2004; Kaech et al., 2003). These studies demonstrate that IL-7Rα^{hi} cells are the memory cell precursors, and provide immunological protection. In addition, IL-7 and IL-7Rα are required for memory cell precursor survival.

Effect of IM on OT-1 Expansion

Decreases in the proliferative potential of OT-1 cells could provide for another explanation of a reduced OT-1 memory response. During the primary response, IM-treated and control CFSE labeled OT-1 cells that were adoptively transferred to B6.Thy1.1 hosts, expanded similarly. Further, adoptive transfer of untreated CFSE labeled OT-1 cells into IM-treated and control hosts also showed a similar expansion. These correlate with an unaltered primary response, and indicate that IM does not affect the *in vivo* expansion of primary OT-1 cells. Moreover, IM did not affect expansion of memory OT-1 cells. The percentage of OT-1 T cells from both IM-treated and control mice that incorporate BrdU during the recall response was also similar. This suggests that an increase in apoptosis is a contributing factor to the lower number of OT-1 cells recovered 6 days later in IM-treated mice. These results are also consistent with our *in vitro* data that show IM at concentrations >5μM induces apoptosis of antigen-stimulated memory cells. Signaling through the IL-7R has been shown to induce synthesis of anti-apoptotic molecules such as Bcl-2, and to block the pro-apoptotic proteins Bad and Bax by post-translational mechanisms (Kim et al., 2003). This could be one mechanism by which a lower level of IL-7Rα on IM-treated memory cells could lead to increased apoptosis and, therefore, a depressed recall response. A tunnel assay which distinguishes apoptotic DNA fragmentation in IM-treated versus control animals would lend evidence for the contribution of apoptosis to the impaired memory OT-1 response. A high DNA fragmentation stain, along with a lower BrdU accumulation, in addition to presence of PI

staining indicate that the lower memory response is due to apoptosis, and not a lower level of expansion.

Direct or Indirect Effects on OT-1 cells

An interesting question is whether the effect of IM on the secondary CD8 T cell response is direct on CD8 T cells or indirect on APC or CD4 cells. However, due to difficulties with generating a TCR transgenic memory population inside the original TCR transgenic host, studies on direct effects on OT-1 cells have their limitations. Furthermore, studies on effects through APC populations are also complicated by the fact that macrophages and DCs both are involved early in the LM infection, and early inflammatory responses influence the CD8 T cell programming (Kaech et al., 2002b; Gett and Hodgkin, 2000; Pamer, 2004; Serbina et al., 2003b). However, several rationales with limitations, can be examined. One method to test the direct effect of IM on CD8 T cells - in the absence of DC and CD4 cells - is the adoptive transfer of effector OT-1 cells into MHC II deficient mice treated with diphtheria toxin (DPT). Delivery of DPT allows for the inducible, short term ablation of DCs *in vivo* (Jung et al., 2002). To generate donor IM-treated OT-1 effectors, the donor OT-1 mice receive a primary LM infection along with IM delivery. Then the OT-1 T cells are adoptively transferred early after the primary response, when they are effectors. The recipients of the effector OT-1 cells receive IM and a secondary high dose LM-OVA infection. The recipients include MHCII KO mice treated with DPT, MHCII KO mice, and wild type mice. This system, however, has its limitations. DCs play an important role during the inflammatory

response to LM. In addition the early inflammatory responses are important for T cell programming, and CD4 help early, either during priming or immediately after, is important in generation of memory response. For these reasons, the best time to transfer the OT-1 cells is when they are effectors, early after the primary response.

CD4 T cell help to Memory CD8 T cells

CD4 cells are not required for the development of efficient primary CD8 T cell responses against infectious agents. However, without CD4 T cells, memory CD8 T cells produce a poorer secondary response than do memory CD8 T cells generated with CD4 help (Sun and Bevan, 2003; Shedlock and Shen, 2003; Janssen et al., 2003; Bourgeois et al., 2002a). The role that CD4 T cells play in the CD8 secondary anti-LM response is not settled. Reports show that CD4 T cells are crucial only during the initial priming of naïve CD8 T cells to differentiate into functional CD8 memory cells (Bourgeois et al., 2002a; Janssen et al., 2003; Shedlock and Shen, 2003; Marzo et al., 2004). In contrast, studies utilizing adoptive transfer of effector or memory CD8 T cells into MHC II deficient mice indicate that CD4 T cell help for the CD8 memory response is essential and plays a role following the resolution of infection, immediately after the early programming phase (Sun et al., 2004). LM-specific CD8 T cells with the potential to undergo extensive proliferation after antigen re-challenge are present early after the primary infection (Busch et al., 2000). Lack of CD4 help compromises the development of these early memory cells (Huster et al., 2004).

Thus, it is possible that the effect of IM on the generation of memory CD8 T cells is partly mediated through its effect on CD4 T helper activity. IM inhibits activation and proliferation of CD4 T lymphocytes *in vitro*, suppresses CD4 T cell cytokine secretion, and modulates a shift towards a T_{H2} response (Cwynarski et al., 2004; Gao et al., 2005; Tsuda and Yamasaki, 2000). In addition, because IM influences antigen-presenting functions of DC, the effect of IM on APC may compromise CD4 T helper activity as well as have a direct effect on CD8 T cell function (Appel et al., 2004; Appel et al., 2005; Taieb et al., 2004). The absence of CD4 T cell help compromises the emergence of functionally competent IL-7Rα^{hi} memory cells, which is consistent with our findings of lower IL-7Rα expression on OT-1 CD8 memory T cells in IM-treated animals (Fuller et al., 2005). Our data indicate that CD4 T cell percentages are not affected by IM during the primary and memory responses. However, since the total number of splenocytes decrease in presence of IM, CD4 total numbers also decrease. Our measurement of the CD4 cells response in this system is based on percentage of cells. In such a system that is directed at studying CD8 T cells, a small change induced by IM on CD4 T cell percentages may not be detectable. The question of CD4 T cell help would be better addressed using a CD4 TCR transgenic system. There is, however, a quantitative decrease in the ability of CD4 T cells during the memory response to produce IFN-γ. This could point to a functional defect in CD4 T cell help. Since the CD4 help has been shown to be important to CD8 memory generation and maintenance, assessing the quality of CD4 help during or right after the primary response is important.

In this model, where CD8 OT-1 cells are adoptively transferred into Thy1.1 hosts, a better investigation on the CD4 T cell status could shed some light on the CD4 help issue. The functional potential of CD4 T cells (IFN-γ) could be examined. In that IM delivery in patients causes a shift towards the T_{H2}, T_{H1} and T_{H2} cytokine patterns by CD4 cells could also be delineated (Gao et al., 2005). However, a more interesting system to study CD4 T cell help utilizes the secondary adoptive transfer of memory OT-I cells along with memory OT-II cells (specific for MHC II epitope of OVA) into B6 (Thy1.2) recipients. The donor OT-I and OT-II cells in the transfer system are on B6.Thy1.1 background (referred to as OT-1.PL and OT-II.PL, respectively) (Hamilton et al., 2006). In this system, both naïve OT-I.PL cells and naïve OT-II.PL cells are adoptively transferred into separate B6 recipients, which receive a primary LM-OVA infection. To test the effect of IM on CD4 cells, only recipients of OT-II.PL cells are treated with the drug. The OT-I.PL recipients remain untreated. After thirty days, the memory OT-I.PL and the memory OT-II.PL cells are purified from spleens of respective recipients. At this point, they undergo a secondary adoptive transfer into B6 recipients. The secondary B6 recipients receive a high dose LM-OVA infection. A bacterial clearance assay on day 1 or 2 after secondary transfer would indicate if the treatment of OT-II.PL memory cells with IM had any effect on the ability of memory OT-1.PL cells to clear the LM. Further, functional quality of CD4 cells can be assessed by IFN-γ or IL-2 staining of CD4 OT-II.PL cells.

CD40-CD40L interactions play an important role in generation of a memory CD8 response. The expression of CD40 molecule on APC and its engagement with CD40L on CD4 cells, delivers help both through APC to the CD8 T cell or by secretion of factors by

CD4 cells. Further, reports indicate that CD40 is also expressed on CD8 T cells to deliver direct signals from engagement with CD40L on CD4 cells. Reports indicate the expression of CD40 molecule on CD8 T cells is important for memory generation (Bourgeois et al., 2002a). However, this is contradicted by others reporting a functional CD8 memory response in absence of CD40 (Sun and Bevan, 2004). In the secondary adoptive transfer system of memory OT-1.PL and OT-II.PL cells into B6 recipients, the contribution of CD40 signaling, either on CD8 or CD4 cells can be examined. This is accomplished by using primary B6 recipients, for both OT-I.PL and OT-II.PL cells respectively, that are deficient in CD40.

The effect of IM on the function of OT-1 cells

One potential implication of a reduced memory response is a functional impairment. When IM-treated LM-OVA immune mice were challenged with a lethal dose of bacteria, we found that they cleared the infection as well as control animals even though the OT-1 CD8 response was impaired. Since LM induces a robust response, it is possible that some of the host anti-LM CD8 T cells were not affected by IM or that other components of the adaptive response compensated for the decreased OT-1 T cell response. The question of function of the specific memory CD8 T cells is better addressed in a secondary adoptive transfer system. Memory OT-1.PL mice will undergo a secondary transfer into IFN-γ deficient B6 recipients receiving a lethal dose of LM, in presence or absence of IM. A bacterial clearance assay on secondary hosts on days 1 or 2 determines if the function of OT-I.PL cells is altered in absence of endogenous memory CD8 T cells.

By using IFN-γ deficient secondary recipients, the contribution of memory CD8 T cells that are activated nonspecifically and contribute to innate defense is circumvented (Berg et al., 2003).

Some CD8 effector mechanisms have more of an impact in specific pathogenic infections. While IFN-γ secretion is the only requirement for CTL clearance of *T. gondii*, IFN-γ is dispensable to the CTL clearance of the LM infection (Gazzinelli et al., 1991; Harty et al., 1992). In addition, during the primary and secondary responses, different mechanisms could contribute to CTL cytolytic activity. For example, perforin has a large contribution during the secondary LM response, and not during the primary response (Kagi et al., 1994b). Furthermore, specific CTL cytolytic activity can contribute to tumor control. For example, tumor surveillance is decreased in perforin deficient mice (van den Broek et al., 1996). In light of these, the question remains that does IM affect a specific CTL cytolytic pathway. In our bacterial clearance assays, we did not observe any evidence indicating one pathway maybe more affected than another. In the context of LM response, chromium release assays (CTL) could determine if a cytolytic pathway is affected more than another. The effectors of such a CTL assay are derived from perforin deficient or Fas deficient OT-1 TCR transgenic mice.

The main CTL effector functions are the production of IFN-γ, and lysis by FasL and/or granzymes. The production of IFN-γ is linked to activation of APCs and generation of more potent cellular responses. The effect of IFN-γ on CD8 T cells is mediated through IFN-γ receptor signals which lead to activation of transcription factor T-bet. T-bet then induces expression of the effector IFN-γ, perforin and granzyme B by

the CD8 T cell (Svensson et al., 2005). In case of an LM infection, production of IFN-γ early in innate response controls the contraction phase and regulates the CD8 T cell response (Haring et al., 2005; Badovinac et al., 2004). The CD8 T cell response to LM infection, however, is not dependent on IFN-γ. This is demonstrated by the fact that CD8 T cells clear the LM infection in IFN-$\gamma^{-/-}$ mice (Harty et al., 1992). In spite of this, IFN-γ influences the magnitude of class Ia response, in that in absence of IFN-γ, the LM specific CD8 T cell response increases (Badovinac and Harty, 2000). Moreover, IFN-γ is important for control of tumors, certain viral infections such as an LCMV infection, and for BMT for hematological malignancies (Pure et al., 2005; Rubio et al., 2005; Terwey et al., 2005; Ritchie et al., 2005; Eliopoulos et al., 2005). While the efficacy of CD8 T cells to mediate tumor rejection correlates with IFN-γ production, IFN-γ secreted by alloreactive T cells contributes to GVHD (Barth, Jr. et al., 1991; Ritchie et al., 2005; Becker et al., 2001). In light of these, our investigations on the quality of OT-1 cells by assessing the IFN-γ production indicate that OT-1 cells are not functionally depressed during either the primary or secondary response. Although the secondary response is reduced in presence of IM, there is not a qualitative change in these memory cells. This is further supported by our *ex-vivo* IFN-γ analysis.

CD8 T cell effector functions can be further examined in a secondary adoptive transfer of memory OT-1.PL cells into IFN-γ deficient B6 recipients, in absence or presence of IM. In the case of IFN-γ, there would be no contributions from host CD8 T cells. Fas L pathways could be examined by staining for FasL upregulation on memory OT-1 T cells. The granzymes/perforin pathway could be tested in memory OT-1 T cells with a flow

cytometric assay for degranulation which utilizes the expression of CD107 on granules (Betts et al., 2003). Other adoptive transfer models that can test the specific CTL effector functions are adoptive transfer of OT-1.PL cells into IFN-$\gamma^{-/-}$, perforin $^{-/-}$ and $FasL^{-/-}$ murine models. These experiments together can delineate what factors contribute to the lower memory CD8 response induced by IM, in addition to what functional consequences this depressed CD8 memory response has.

Influence of IM on TCR/Abl Signaling

The expression of IL-7Rα is high on naïve cells, while it is downregulated on antigen activated cells and re-expressed again on memory cells. It is likely that the expression of IL-7Rα on mature T cells is regulated by a different mechanism than that in thymocytes. Further, it is likely that the regulation of IL-7Rα is different in mature naïve T cells versus activated or memory T cells. Mature naïve CD8 T cells are dependent on IL-7 and MHC-peptide contact for survival. Therefore, one would expect that naïve cells are very responsive to IL-7, and both IL-7 and the affinity of MHC-peptide interaction be determinants of the high IL-7Rα expression on naïve cells. In fact, investigations indicate that both IL-7 and the affinity between TCR and the peptide-MHC complex regulate IL-7Rα expression (Ma et al., 2006; Schluns et al., 2000). However, activated T cells may not be very responsive to IL-7. Therefore, an activated CD8 T cell would not depend on IL-7 for survival. It is likely that the regulation of the promoter of IL-7Rα therefore is regulated differently in activated versus naïve CD8 T cells. In contrast, memory cells depend on both IL-7 and IL-15 for survival and maintenance (Kieper et al.,

2002; Tan et al., 2002). This could explain why IL-7Rα expression is high on memory cells, but not as high as that in naïve cells.

Signaling through IL-7Rα has been linked to expression of the anti-apoptotic factor Bcl2 (Kim et al., 1998; Akashi et al., 1997). In addition, the IL-7 signaling cascade may be responsible for regulation of an immediate-early transcription factor, Kruppel-like Factor 2 (KLF2) (Schober et al., 1999; Kuo et al., 1997). KLF2 (also called LKLF) is necessary for the maintenance of naïve T cells. It is expressed in both naïve and memory cells, and is proposed to promote quiescence in these populations (Endrizzi and Jameson, 2003). In activated cells, KFL2 expression is rapidly down-regulated as both the mRNA and protein levels decrease in activated cells. However, it has been suggested that protein degradation of KFL2 is delayed during the very early stages of T cell activation to allow for control of IL-2 expression. This rapid upregulation during the early stages of T cell activation leads to a rapid increase in IL-2 production from the activated cells (Wu and Lingrel, 2005). Within a few hours of activation, KFL2 expression is turned off (Wu and Lingrel, 2005). This downregulation of KFL2 is critical for full T cell activation (Endrizzi and Jameson, 2003). While IL-7 signals promote KLF2 re-expression in antigen-stimulated cells, they are not required for KLF2 maintenance in naïve T cells (Endrizzi and Jameson, 2003). This suggests that IL-7 signals have distinct effects on KLF2 expression in naïve verses activated T cells. Since the roles of the IL-7Rα signaling cascade are different in naïve versus activated cells (as shown in relation to KFL2), it is likely that the expression of the IL-7Rα itself is regulated differently.

In this study, the administration of IM decreases the secondary OT-1 T cell response as well as decreasing the percentage of cells expressing high levels of IL-7Rα. A question that remains mechanistically is why are the IL-7Rα^{hi} cells not present in IM-treated animals? It is known that IL-7 deprivation of cells leads to metabolic arrest, decreased cell size, reduced glycolysis, and delayed mitosis in response to stimuli *in vitro* (Rathmell et al., 2001). IL-$7^{-/-}$ and IL-7R$\alpha^{-/-}$ mice exhibit severe lymphoid hypoplasia, including deficiencies of both B and T lymphocytes (von Freeden-Jeffry et al., 1995; Peschon et al., 1994). IL-7 primarily affects T cell development by maintaining the viability of precursor cells independent of cell division (Hofmeister et al., 1999), and by promoting V(D)J recombination (Hofmeister et al., 1999).

Furthermore, IL-7 also mediates the survival of naïve and memory cells *in vivo* (Schluns et al., 2000). By using the transfer of OT-1 $RAG^{-/-}$ (Ly5.2) T cells into irradiated IL-$7^{-/-}$ and IL-$7R^{-/-}$ Ly5.1 congenic hosts followed by immunization with vesicular stomatitis virus expressing ovalbumin, schluns et al showed that IL-7R is regulated during T cell activation and memory induction. Further transfer of OT-1 IL-$7R^{-/-}$ T cells into normal B6 mice followed by infection showed that IL-7R is essential for $CD8^+$ memory T cell production (Schluns et al., 2000). The link between expression of IL-7R and apoptosis is further demonstrated by data showing re-expression of Bcl-2 is partially impaired in IL-7R deficient OT-1 T cells. IL-7Rα expression is preserved on a subset of activated $CD8^+$ T cells that progress to become memory $CD8^+$ T cells (Schluns et al., 2000; Goldrath et al., 2002; Kaech et al., 2003). In particular, IL-$7R^{-/-}$ OT-1 CD8+ T cells, survive poorly as memory CD8+ T cells (Schluns et al.,

2000). Signaling through IL-7R activates Jak kinases (Jak1 and Jak3), leading to activation of STATs, phosphoinoside-3 kinase, Ras and MAPK/ERK (Hofmeister et al., 1999; Leonard et al., 1999).

Since IL-7Rα expression is regulated at the level of transcription, the *cis*-control elements of the IL-7Rα locus have been analyzed (Lee et al., 2005). Analysis of the IL-7Rα promoter has shown a GGAA motif which can be used as binding sites for GA binding protein (GABP) (Xue et al., 2004; Lee et al., 2005). The promoter region also contains a functional interferon regulatory element, and potential binding sites for AP-1 and AP-2 and multiple glucorcorticoid response elements (Lee et al., 2005; Pleiman et al., 1991). Comparison of mouse, rat and human IL-7Rα loci revealed striking homology in a region about 320 bp upstream of the translation initiation site (the transcription initiation site is 46 and 130 bp upstream of the translation initiation site) (Lee et al., 2005). Consensus motifs of PU.1, Runx1 (both transcription factors for lymphocyte progenitors), and Ikaros were conserved in the region upstream of translation initiation site (Lee et al., 2005). In addition a noncoding sequence (CNS) has been identified upstream of the IL-7α promoter which activates the transcription of the IL-7Rα promoter (3.6 kb upstream of the translation initiation site) (Lee et al., 2005). The percent homology of the CNS region was 86% between mouse and human, and included consensus motifs for GATA, NF-κB, GR and Evi-1 transcription factors. Of the above motifs the PU.1, Runx1, Ikaros in the promoter region have been tested for transcriptional activity. Data indicates the PU.1 motif, and possibly Runx motif are important for activity of IL-7Rα promoter. In addition, only the GR motif in the CNS region has been

tested and shown to activate the transcription of the IL-7Rα promoter (Lee et al., 2005). The other conserved consensus motifs have not been tested for transcriptional activation of the IL-7Rα promoter.

c-Abl deficient mice show a variety of phenotypes including reduced T cell numbers and increased susceptibility to infection (Schwartzberg et al., 1991; Tybulewicz et al., 1991). These phenotypes indicate that Abl, which can be myristoylated, could have a function in T cells. In fact, TCR/Abl signaling has been reported to be suppressed with IM, leading to lack of phosphorylation on signaling molecules involved in TCR/Abl signaling pathways (Zipfel et al., 2004). In Jurkat T cells, Abl is activated in an Lck-dependent manner (Zipfel et al., 2004). Abl kinases are required for signaling through TCR on primary Abl deficient cells. Inhibition of Abl kinases by IM leads to a stop in proliferation and IL-2 production in response to TCR stimulation on primary T cells from $Abl1^{+/-}$ $Abl2^{+/-}$ and $Abl1^{+/-}$ $Abl2^{-/-}$ mice (Zipfel et al., 2004). Specifically, Zap70 and LAT, molecules involved in TCR signaling are phosphorylated by Abl, and phosphorylation on LAT and downstream Erk is inhibited in the presence of IM (Zipfel et al., 2004). Transcription reporter assays show downstream transcriptional activation of IL-2, CD28RE/AP and NFAT by TCR/Abl are inhibited by IM. This study establishes a link between TCR signaling, Abl and transcriptional activation.

Based on the above studies, I hypothesize that the expression of IL-7Rα is downregulated through the effects of IM on TCR signaling. In presence of IM, signaling through TCR/Abl decreases. Abl can be found in a myristoylated form loosely attached to the inner surface of cell membrane. Upon TCR stimulation, Abl is activated in an

Lck-dependent manner. IM interferes with phosphorylation and activation of Abl by binding to its kinase pocket. Abl phosphorylates ZAP70. A block in phosphorylation of Abl causes a decrease in phosphorylation of ZAP70 leading to a decrease in phosphorylation on LAT. This results in a block in NF-κB translocation. In addition a decrease in phosphorylation of Erk could block the translocation of AP-1 to the nucleus. Since AP-1 or NF-κB presumably control IL-7Rα promoter activation, IL-7Rα expression decreases in the cell, leading to a decrease in IL-7Rα molecules on the CD8 T cell surface, and hence the disappearance of IL-7Rα^{hi} cells in presence of IM.

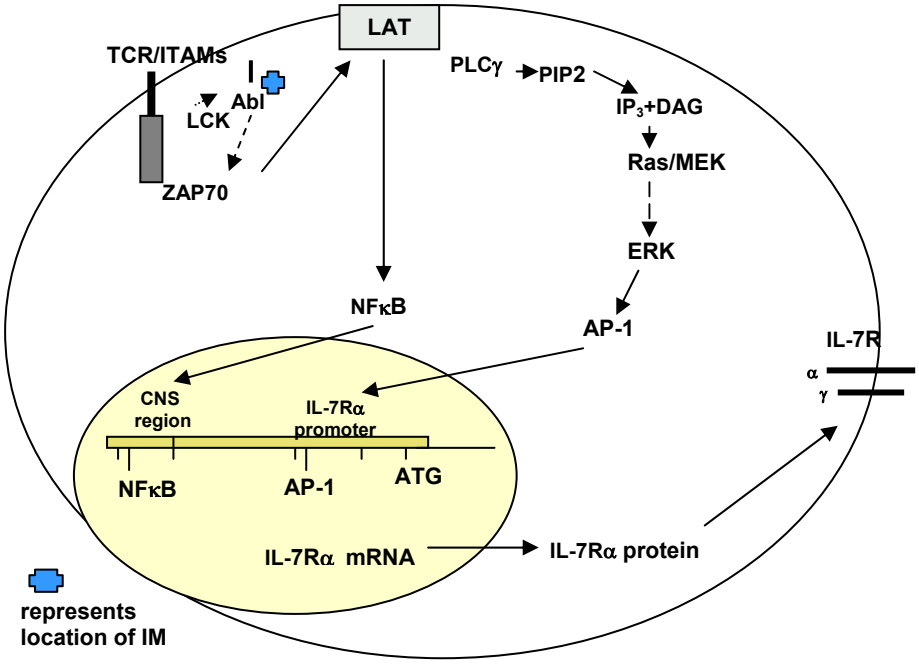

Figure 1. Schematic of hypothesis stated.

In order to test if modulations in TCR/Abl signaling lead to changes in IL-7Rα expression on the cell surface, I would use Abl deficient, wild-type, and Abl deficient reconstituted with Abl (refer to as Abl deficient/Abl reconstituted) primary T

cells stimulated with anti-CD3. I would expect a decrease in IL-7Rα expression as the cells are activated. However, in absence of Abl, there should be a further decrease in IL-7Rα levels. Reconstitution of Abl deficient cells with Abl should rescue this decrease to the IL-7Rα levels in wild-type activated cells.

Further, to test the involvement of NF-κB or AP-1 transcription factors I would construct the consensus binding sequence of the transcription factor in question next to a reporter gene such as luciferase. Upon stimulation of Abl deficient/Abl reconstituted cells, I expect higher levels of luciferase activity for NFκB/ or AP-1. To show that the transcription factor is binding to its consensus sequence in the CNS region upstream of IL-7Rα promoter or the IL-7R promoter itself, I would do a gel shift assay. I would digest the genomic DNA of wild type cells to get the appropriate size for the consensus binding sequence in the IL-7Rα promoter region. The digest is run on a gel and the size corresponding to consensus sequence in the IL-7Rα promoter region is purified and labeled with P^{32}. Then, nuclear extracts of Abl deficient/Abl reconstituted cells, and control wild type and Abl deficient cells are prepared. All these cells have been activated with anti-CD3 prior to harvest. The P^{32} labeled consensus sequence from the IL-7Rα promoter is incubated with the different nuclear extracts (indicated above). The reaction products are analyzed on a nondenaturing polyacrylamide gel. Binding of the transcription factor to its consensus motif in the promoter region should yield a shift corresponding to the DNA-protein complex on the gel (this is expected in the protein-DNA samples prepared from wild type and Abl deficient/Abl reconstituted cells, and not from samples prepared from Abl deficient cells).

The implications of a depressed memory response

The inhibitory effects of IM on memory CD8 T responses have important clinical implications. In CML patients, there are case reports of increased infections in patients undergoing IM treatment (Ikeda et al., 2006; Lin et al., 2004). Furthermore, a reactivation of herpes zoster infection has been observed in CML patients treated with IM (Mattiuzzi et al., 2003). This could be the result of a defective CD8 memory response and would be consistent with the effect we observed with OT-1 T cells. Although we did not observe an impairment of function associated with the depressed memory response, the function could be impaired if pathogen levels are increased. An increase in pathogen levels correlates with antigen levels of very infectious agents.

However, one should also note the beneficial effects of IM on viral infections. Recent studies demonstrate that Abl kinase, in conjunction with Src family kinases, stimulates actin-based motility of vaccinia virus (Newsome et al., 2006; Reeves et al., 2005). The inhibition of Abl by IM, therefore, reduces vaccinia viral dissemination and promotes survival in mice (Reeves et al., 2005). In addition, the inhibition of PDGF and c-kit activity by IM may induce regression of AIDS-related Kaposi's Sarcoma (Koon et al., 2005). Thus, while regression of viral infection is a positive aspect of IM therapy, the immunosuppressive effects of IM, such as interference with memory CD8 responses is a negative factor that could interfere with responses against leukemia or infectious pathogens.

Further, recent clinical trials use high dose IM (800mg) to treat patients. This high dose is expected to produce higher levels of IM in plasma (Rea et al., 2006; Kantarjian et al., 2004; Druker et al., 2001; Peng et al., 2004b). High IM could have a more profound suppression of the CD8 response. In OT-1 adoptive transfer system, however, we cannot delineate the effects of high IM administration to B6 mice. IM dosage studies in the B6 model indicated these animals could not tolerate high levels of IM for the extended period of time it is needed to generate a memory response.

Allogeneic bone marrow transplantation plays an important role in the management of CML (Hansen et al., 1998). IM therapy is used to treat relapse after allogeneic bone marrow transplantation (Hess et al., 2005; Kantarjian et al., 2002; Olavarria et al., 2003; DeAngelo et al., 2004). DLIs are often administered for relapsed allogeneic BMT in an effort to induce GVL. The effect of IM on CD8 T cells in GVL reactions is not known and it is possible that IM could compromise this activity along with increasing susceptibility to certain opportunistic infections.

Future directions

The studies presented here have provided an understanding of the immunosuppressive effects of IM on antigen specific memory CD8 T cells. Adoptive transfer of TCR transgenic OT-1 T cells (Thy1.2$^+$) into a Thy1.1$^+$ recipient has allowed for direct assessment of the effect of IM on antigen specific CD8 T cell responses. Previous studies focused on the inhibitory effects of IM on T cells *in vitro*. We demonstrated that IM lowers the memory CD8 T cell response. While IM did not influence the primary antigen

specific CD8 response, the events early on contributed to the depressed secondary response. This was demonstrated by tracing the memory cells with the memory marker, IL-7Rα, from the peak of primary response to after a second encounter with antigen. Furthermore, the quality and function of memory CD8 T cells at this low antigen dose was not influenced. Moreover, the hypothesis that the impaired CD8 memory response is due to lack of CD4 help is supported by the fact that functionally CD4 T cells are impaired during the memory response.

As previously discussed, much still remains unclear about the mechanisms that contribute to the lower memory responses. It would be of interest to delineate whether this effect is direct on memory CD8 T cells or indirect through DCs or CD4 cells. Likely, it is a combination effect. This has implications for immunotherapy of CML patients under treatment with IM, or those patients who relapsed after allogeneic BMT, and are receiving IM and donor lymphocyte infusions.

Furthermore, it remains unclear what the functional consequences of the lower CD8 memory responses are. The effect of IM on the function of CD8 T cells is an important determinant of pathogen clearance. The surfacing of latent viral infections, particularly herpes viral infection, has been observed in CML patients under treatment with IM. In addition, in allogeneic transplant recipients, who are immunocompromised as result of chemotherapy and radiation therapy, the occurrence of infections can be detrimental. Assessing for the IFN-γ cytokine or clearance of LM from the spleen and liver indicate that there are not functional consequences. However, this likely will not hold true if the antigen dosage, TCR avidity or IM dosage is increased. In fact, patients are treated with

high dose IM. In the LM model, it would be of interest to give these animals higher levels of LM to determine whether a functional defect will be detected. TCR avidity and IM dosage can be tested in a different pathogenic model using other TCR transgenic cells. Adoptive transfer of P14 TCR transgenic cells into recipients infected with LCMV provides for a different model to this respect.

Immune responses to bacteria differ from those to a virus. There are studies that indicate CML patients under treatment with IM develop herpes zoster infections. It will be of interest to determine whether IM influences the CD8 T cell responses to a virus, preferably one that is pathogenic to humans. While LCMV is a good model for studying a chronic infection, vaccinia virus or hepatitis B virus are more pathogenic to humans. Either of these systems provides a better understanding of the influence of IM on CD8 cells and effector mechanisms to a virus. This has implications in latent viral infections experienced by some CML patients under treatment with IM.

Immune responses to a tumor differ from those to bacteria. While it is possible that patients under treatment with IM may have higher incidences of infections, it is unclear how the CML specific tumor response is influenced by IM. Patients in the chronic phase of CML under therapy with IM show remissions because of the effects of IM on ph^+ cells, not on T cells. A possible explanation for why CML progresses regardless of the presence of tumor specific T cells, is tolerance. In addition, tumor burden and lack of antigen specific T cells early in tumor response may also contribute. To gain an insight on the status of OT-1 cells in a CML murine model under treatment with IM, the bone marrow transduction/transplantation CML murine model can be used. Transplant of

BCR-ABL infected OT-1 bone marrow into irradiated B6 recipients that are treated with IM provides for a model where antigen specific T cells can be traced with use of the appropriate tetramer. These studies have direct implications in augmenting CD8 specific tumor response in CML.

CD8 T cells have been implicated to play a role during the GVL response. It is of interest to determine what effect IM has on CD8 T cells in a BMT model. There are murine BMT models available to study either CD4 or CD8 responses in GVL in a CML setting (Krause and Van Etten, 2004; Matte et al., 2004). If such models can be adapted to induce a TCR transgenic CD8 T cell response, then such a model would provide for a characterization of the effects of IM on CD8 T cells in GVL. These studies will further impact immunotherapy to induce GVL in CML patients.

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VITAE

Parisa Sinai was born in Tehran, Iran, on April 9, 1970, the daughter of Ali and Faranik F. Sinai. In 1983 she moved to Sunnyvale, California and lived with her brother. She graduated from Homestead High School in 1988. She received her Bachelor of Arts in majors of Molecular and Cell Biology, and Near Eastern Studies in June 1993 from the University of California, Berkeley. After graduation, she worked for Clontech Laboratories, Inc, Palo Alto, California in the department of Cell Biology. In 1999, she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas, and was admitted as a candidate in the Immunology Graduate Program in 2001. After completing her studies in the laboratory of Dr. James Forman, she was awarded a Doctor of Philosophy degree in September, 2006. In 1997, she married Christoph Wuelfing. Her daughters, Chiara S. Wuelfing and Mara S. Wuelfing, were born in 2002 and 2004, respectively.

Permanent Address: 3629 Herschel Ave.

Dallas, TX 75219