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Defining a T Cell-Intrinsic Role for MyD88 During LCMV infection

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Abstract
Immune activation through Toll-like receptors (TLRs) has historically been considered to be a characteristic of cells of the innate, rather than adaptive immune system. Recent studies have challenged this paradigm by demonstrating that TLRs are also expressed on T lymphocytes and that TLR ligands can directly co-stimulate T cell responses in vitro. However, the physiological relevance of these findings during in vivo immune responses was unclear. Mice lacking the critical TLR-adapter protein, myeloid differentiation protein 88 (MyD88), have increased susceptibility to numerous pathogens, highlighting the importance of TLRs in host defense. While the immune impairments associated with MyD88-deficiency have generally been attributed to the importance of MyD88 in regulating innate immune responses, in light of the studies showing that TLRs can directly stimulate T cells, we hypothesized that they may also reflect a direct role for MyD88 in T cells. In this work, we use lymphocytic choriomeningitis virus (LCMV) as a model infection to examine the role of MyD88 in regulating antiviral T cell responses. Using a series of adoptive cell transfer and bone marrow chimera experiments, we identify a critical, but previously unappreciated, T-cell intrinsic role for MyD88 in regulating the survival and expansion of LCMV-specific effector T cells during acute viral infection. Using a system to inducibly delete MyD88 we also show that, while naïve T cells critically depend on MyD88-dependent signals for their expansion, virus-specific memory T cells do not require MyD88 for their differentiation, maintenance or reactivation in response to secondary infection. Overall, our findings broaden the importance of MyD88 in T cells, support a shift in the dogma that restricts the role MyD88 to cells of the innate immune system, and may have significant implications for understanding the signals that control T cell survival during inflammatory immune responses.

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ABSTRACT

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Adeeb Habibur Rahman

Laurence Turka, M.D.

Immune activation through Toll-like receptors (TLRs) has historically been considered to be a characteristic of cells of the innate, rather than adaptive immune system. Recent studies have challenged this paradigm by demonstrating that TLRs are also expressed on T lymphocytes and that TLR ligands can directly co-stimulate T cell responses *in vitro*. However, the physiological relevance of these findings during *in vivo* immune responses was unclear. Mice lacking the critical TLR-adapter protein, myeloid differentiation protein 88 (MyD88), have increased susceptibility to numerous pathogens, highlighting the importance of TLRs in host defense. While the immune impairments associated with MyD88-deficiency have generally been attributed to the importance of MyD88 in regulating innate immune responses, in light of the studies showing that TLRs can directly stimulate T cells, we hypothesized that they may also reflect a direct role for MyD88 in T cells. In this work, we use lymphocytic choriomeningitis virus (LCMV) as a model infection to examine the role of MyD88 in regulating antiviral T cell responses. Using a series of adoptive cell transfer and bone marrow chimera experiments, we identify a critical, but previously unappreciated, T-cell intrinsic role for MyD88 in

regulating the survival and expansion of LCMV-specific effector T cells during acute viral infection. Using a system to inducibly delete MyD88 we also show that, while naïve T cells critically depend on MyD88-dependent signals for their expansion, virus-specific memory T cells do not require MyD88 for their differentiation, maintenance or reactivation in response to secondary infection. Overall, our findings broaden the importance of MyD88 in T cells, support a shift in the dogma that restricts the role MyD88 to cells of the innate immune system, and may have significant implications for understanding the signals that control T cell survival during inflammatory immune responses.

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CHAPTER I: Introduction and Overview

Introduction

The immune system can broadly be divided into two arms: the innate and adaptive. The innate immune system serves as a first line of defense against pathogens and also plays an important role in initiating and directing adaptive immune responses. Cells of the innate immune system, such as neutrophils, macrophages and dendritic cells, recognize foreign antigens through a limited range of germ-line encoded receptors, collectively known as pattern recognition receptors (PRRs)(Janeway & Medzhitov 2002). These PRRs recognize highly conserved molecular motifs that are generally found in microbial pathogens, but not in their eukaryotic hosts and are thus termed pathogen-associated molecular patterns (PAMPs). In contrast, cells of the adaptive immune system, such as B and T lymphocytes are activated through antigen receptors that are generated by genetic rearrangement and selected to recognize an incredibly diverse array of potential ligands(Oettinger et al. 1990). Thus, activation through PRRs, versus rearranged antigen receptors, has historically been considered a defining feature that distinguishes cells of the innate arm from those of the adaptive arm of the immune system.

Toll like receptors

Several classes of PRRs have been now described, but Toll like receptors (TLRs) represent one of the best characterized. TLRs are a family of evolutionarily conserved receptors that have a broad, heterogeneous tissue expression pattern, and whose ligands

can mediate effects on a variety of cell types. The TLR family currently includes at least 11 members that bind to a range of microbial components from bacteria, fungi and viruses. TLRs 2, 4, 5 and 11 are expressed on the cell surface and recognize bacterial lipoproteins, lipopolysaccharide (LPS), flagellin and profilin respectively. TLRs 3, 7, 8 and 9 are expressed in endosomal compartments and are involved in the recognition of double stranded RNA, single stranded RNA, and unmethylated CpG DNA, respectively. These details of TLR ligand-recognition and cellular localization are summarized in Table 1. The molecular mechanisms that allow for the broad diversity in TLR ligand recognition remain unclear and recent studies of the structure of TLR receptor-ligand complexes suggest that different TLRs may employ different ligand-binding interfaces(Jin et al. 2007)(H. M. Kim et al. 2007)(L. Liu et al. 2008).

While TLRs have generally been considered a means to distinguish "non-self" from "self", it is interesting to note that endogenous molecules may also act as TLR ligands. In analogy to PAMPs, these putative endogenous ligands may be considered damage associated molecular patterns (DAMPs)(Bianchi 2007). Heat shock proteins (HSPs), and extracellular matrix components such as fibronectin and hyaluronan are released during stressful tissue injury and have been reported to activate the innate immune system through TLR2 and TLR4(Vabulas et al. 2001)(Ohashi et al. 2000)(Okamura et al. 2001)(Termeer et al. 2002), though the potential contamination of these endogenous ligands with PAMPs is a concern in some of these studies(Gao & Tsan 2003a)(Gao & Tsan 2003b). In addition, chromatin complexes released from damaged cells contain nucleic acids that can engage TLR7 and TLR9(Leadbetter et al. 2002)(Lau et al. 2005). Indeed, it

has been suggested that the intracellular localization of these nucleic acid-sensing TLRs restricts their access of to endogenous ligands and thereby contributes to the discrimination between self and non-self nucleic acids(Barton et al. 2006). However, this discrimination is imperfect and, consequently, TLR-mediated recognition of endogenous nucleoproteins has been implicated in the development and progression of autoimmune diseases such systemic lupus erythamtosis (SLE)(Christensen et al. 2006)(Ehlers et al. 2006). These studies highlight the fact that the TLR family plays a role in the recognition of an incredibly broad array of ligands, the overall diversity of which may yet to be fully appreciated.

TLR signaling

TLRs are type I integral membrane glycoproteins that contain a leucine rich repeat motif, which serves as a ligand binding domain, and an intracellular cytoplasmic Toll/interleukin-1 receptor (TIR) domain that initiates signal transduction(Jin et al. 2007)(Jin & Lee 2008). The super-family of TIR-domain containing receptors also includes the IL-1R/IL-18R family(Adachi et al. 1998). These receptors act by recruiting and homodimerizing with TIR domain-containing adaptors proteins. Myeloid differentiation protein 88 (MyD88) is one of several adapter proteins linking these receptors to downstream signaling molecules(Medzhitov et al. 1998). Importantly, MyD88 is required for signaling through all TLRs except TLR3, which signals through the alternate adaptor molecule, Toll/IL-1R domain containing adaptor-inducing IFN-β (TRIF), and TLR4,

which can signal through both MyD88- and TRIF-dependent pathways(Yamamoto et al. 2002)(Yamamoto et al. 2003).

Following interaction with upstream receptors through its TIR domain, MyD88 recruits downstream signaling molecules through its death domain. Homotypic death-domain interactions lead to the activation of IL-1 receptor-associated kinases (IRAKs) and the subsequent activation of TNF receptor-associated factor 6 (TRAF6), ultimately resulting in the activation of NFκB and AP-1(Wesche et al. 1997)(Martin & Wesche 2002). While the nuclear translocation of NFκB is generally considered to be classical outcome of MyD88-dependent signaling pathways, numerous studies have established that there is considerably more diversity and complexity in the potential signaling pathways that can be induced through MyD88. For example, TLR signaling can also activate the Akt pathway through a direct interaction of phosphatidylinositol 3-kinase (PI-3-K) with a tyrosine motif in the MyD88 TIR domain(Li et al. 2003)(Rhee et al. 2006)(Gelman et al. 2006). MyD88 has also been shown to form complexes with interferon regulatory factors (IRF)-1, IRF-5, and IRF-7, which play an important role in the induction of type I interferons (IFN-I) and other proinflammatory cytokines by TLR signaling in APCs (Balkhi et al. 2008a)(Takaoka et al. 2005)(Kawai et al. 2004)(Honda et al. 2005)(Balkhi et al. 2008b)(Honda et al. 2004).

MyD88 has also been found to contribute to pathways outside of its traditional role in TLR signaling. The ability of MyD88 to undergo homotypic interactions with other death-domain containing proteins has been shown to allow it to interact with Fas-associated protein with death domain (FADD), implicating MyD88 as a bridge between

TLR and Fas-mediated signaling pathways(Altemeier et al. 2007)(Zhande et al. 2007). Additionally, MyD88 has been shown to be able to interact with the interferon gamma receptor (IFNγR1) and stabilize IFNγ-induced mRNA transcripts(D. Sun & Ding 2006). Overall, these studies identify MyD88 as a critical regulator of TLR signaling and an important branching point in the diversity of signals and cellular outcomes that can be induced through TLR stimulation and other potential upstream mediators.

TLRs stimulate antigen presenting cell responses

TLRs are highly expressed on antigen presenting cells (APCs) of the innate immune system, such as macrophages and dendritic cells, and have been most extensively studied in these cell types. TLR engagement induces APC maturation, characterized by increased expression of costimulatory molecules such as CD80 and CD86 as well as the production of pro-inflammatory cytokines such as IL-12, IL-6 and type I IFNs(Fearon & Locksley 1996)(Krug et al. 2001). Furthermore, maturation precipitates a shift in adhesion molecule and chemokine expression allowing APCs to traffic to the T cell areas of the spleen and lymph nodes(Michelsen et al. 2001)(Turnbull et al. 2005). TLR engagement also promotes the stability of cognate MHC peptide complexes and upregulates their expression on the surface of APCs(Hertz et al. 2001)(Rudd et al. 2008), and the presence of TLR ligands in phagocytosed antigens has also been shown to promote the generation of peptide:MHC Class II complexes thereby directly enhancing the presentation of phagocytosed antigens(Blander & Medzhitov 2006). On account of these effects, TLR stimulation on APCs allows them to more effectively activate T cells, promoting the proliferation and

differentiation of naïve T cells into effector cells. In the absence of these maturation signals, the ability of APCs to initiate T cell responses is greatly reduced(Pasare & Medzhitov 2004).

TLRs are expressed on B and T lymphocytes

Due to their importance in the antigen presenting cells, it is well established that TLRs also play a key, indirect role in promoting adaptive immune responses. However, recent studies have demonstrated that the expression and function of TLRs is not limited to cells of the innate immune system. Mouse and human B lymphocytes have been found to express several TLRs and TLR stimulation promotes B cell proliferation, differentiation, antibody class switching, cytokine production and survival(Pasare & Medzhitov 2005)(Ruprecht & Lanzavecchia 2006)(Yi et al. 1998). The ability of TLR ligands to modulate B cell responses demonstrates that TLRs can directly contribute to adaptive immune responses. A number of studies have now further extended these findings to show that TLRs are also expressed by various subsets of T lymphocytes.

In two studies, sorted CD4+CD45RB^{high} T cells from C57BL/6 (B6) mice were found to express TLR1, 2, 3, 6, 7 and 8, but low to undetectable levels of TLR 4, 5 and 9 mRNA(Caramalho et al. 2003)(Tomita et al. 2008). Naïve CD4 T cells from BALB/c mice have been shown to express mRNA for TLR3, 4, 5 and 9(Gelman et al. 2004), while naïve CD8 T cells from B6 mice have been reported to express mRNA for TLR1, 2, 6 and 9 but not TLR4(Sobek et al. 2004)(Cottalorda et al. 2006). Regarding protein expression, TLR2, 3, 4 and 9 protein have been reported to be found on murine CD4 T cells via flow

cytometry(Fukata et al. 2008), and TLR2 protein has been detected by flow cytometry on murine CD8 T cells(Sobek et al. 2004)(Cottalorda et al. 2006). Furthermore, human T cells isolated from peripheral blood have also been reported to express mRNA for most TLRs, though there is considerable variation in the reported expression levels in these studies(Hornung et al. 2002)(Mansson et al. 2006)(Zarember & Godowski 2002). Protein expression of TLR2, 3, 4, 5, 9 has also been detected by flow cytometry on human T cells(Mansson et al. 2006)(Crellin et al. 2005)(Komai-Koma et al. 2004)(Wesch et al. 2006).

Stimulation through the B cell receptor has been shown to upregulate TLR expression on B cells, resulting in enhanced sensitivity to TLR ligands(Ruprecht & Lanzavecchia 2006). Similarly, TLR expression on T cells appears to be regulated by T cell receptor-dependent activation. Naïve BALB/c CD4 T cells upregulate TLR3 and TLR9 mRNA in response to T cell receptor (TCR) stimulation by αCD3 antibodies(Gelman et al. 2004). Furthermore, while naïve BALB/c T cells were shown to express intracellular TLR2, expression on the cell surface was only readily detectable by flow cytometry following αCD3-induced activation(Liu et al. 2006). Similarly, B6 CTLs express higher levels of TLR2 mRNA than naïve CD8 T cells(Sobek et al. 2004) and TCR stimulation increases surface expression of TLR2 protein as detected by flow cytometry(Cottalorda et al. 2006). Antigen experienced T cells appear to continue to maintain higher levels of TLR expression since CD4+CD45RB^{low} memory cells have also been shown to express higher mRNA levels of most TLRs than naïve CD4+CD45RB^{high} T cells(Caramalho et al. 2003) and human CD45RO+ memory CD4 T cells express higher levels of TLR2 than naïve T cells(Komai-Koma et al. 2004).

To summarize the above, while TLR expression on various T cell subsets has been independently confirmed by a number of studies, the specific patterns of expression that have been reported vary considerably. Thus there may be significant species, and even strain, specific differences in TLR expression. It is also possible that these differences in expression result from technical variations in different studies. It is also important to note that mRNA levels do not always correlate with protein expression, and TLR expression is known to be regulated at the protein level by ubiquitin-mediated degradation(Chuang & Ulevitch 2004)(Boone et al. 2004). Furthermore, minimal contamination of purified T cells by APCs could significantly influence results. In addition, studies that have examined TLR protein levels in T cells are limited by the reliability of available antibody reagents and personal experience with reagents to detect murine TLRs via flow cytometry has been quite variable. These caveats may partially explain the considerable variability in the TLR expression patterns that have been reported on T cells. Given the limitations in examining expression levels of TLRs on T cells, the ability of known TLR ligands to activate signaling pathways may be a more reliable indicator of expression.

TLRs can act as T cell costimulatory receptors

Effective T cell activation requires a primary signal delivered through the TCR, however this signal alone is insufficient to induce effective T cell activation. Optimal expression of effector cytokines and the synthesis of pro-survival molecules depend on additional secondary signals delivered through co-stimulatory receptors. CD28 is generally

considered to be the principle co-stimulatory receptor involved in initial T cell activation(Sharpe & Freeman 2002) and early studies using CD28 deficient T cells found greatly reduced T cell responses in the absence of CD28 signaling(Lucas et al. 1995). A number of studies have now found that TLR ligands can act directly on highly purified αβ T cells to provide costimulatory signals in the absence of traditional CD28 signaling from APCs.

Conventional CD4 and CD8 T cells

As discussed earlier, mouse and human CD4 and CD8 T cells have been shown to upregulate TLR2 expression as a result of activation through the TCR(Komai-Koma et al. 2004)(Cottalorda et al. 2006). Consistent with the this increased expression, it has been demonstrated that the synthetic TLR2 ligand, Pam_3CysSK_4, can act directly on highly purified B6 CD8 T cells to promote enhanced proliferation and survival associated with sustained CD25 expression(Cottalorda et al. 2006). TLR2 ligands also promote CD8 T cell effector functions such as IFNγ production, granzyme B secretion and cytolytic activity. Costimulation through TLR2 also allows lowers the threshold of activation, thereby allowing CD8 T cells to differentiate into functional memory cells in response suboptimal levels of TCR stimulation(Mercier et al. 2009). TLR2 has similarly been shown to costimulate proliferation and IFNγ production from B6 mouse CD4 T cells(Rahman et al. 2009). While purified CD4 T cells from Balb/c mice were reported to not respond to TLR2, 5 or 7 ligands, Poly(I:C) and CpG-containing oligonucleotides (CpG-ODN), the synthetic ligands for TLR3 and TLR9 respectively, promoted enhanced activated T cell survival(Gelman et al. 2004). CpG-ODN have also been shown to support enhanced

proliferation and IL-2 production from activated B6 CD4 T cells(Gelman et al. 2006)(Bendigs et al. 1999) and have recently also been shown to enhance proliferation of rat CD4 T cells(Chiffoleau et al. 2007).

Similarly to mouse T cells, both naïve CD45RA+ and memory CD45RO+ human CD4 T cells have been shown to directly respond to TLR2 ligands(Komai-Koma et al. 2004). However, in contrast to mouse T cells, synthetic ligands for TLR5 and 7/8 have also been shown to enhance proliferation and cytokine production in human CD4 T cells, particularly in CD45RO+ memory cells(Caron et al. 2005). A recent study has also found that human, but not mouse, CD8 T cells produce IFNγ, perforin and granzyme in response to LPS signaling through TLR4(Komai-Koma et al. 2009). In contrast, another recent study attributes to the costimulatory effect of LPS on human CD4 T cells to APC contamination, but supports a role for direct TLR2 and TLR5 induced signals in promoting cytokine production and reducing the threshold for CD4 T cell activation(Lancioni et al. 2009). Notably, this study highlights the importance of stringent T cell purification methods when studying the direct effects of TLR ligands on T cells since minimal APC contamination is sufficient to significantly alter the results of experiments.

Overall, these studies demonstrate that several TLR ligands can act directly on T cells *in vitro*, though there are clearly species, and even strain specific differences in the ligands that are recognized. It is important to note that these studies consistently show that TLR-induced signals in naïve T cells are strictly co-stimulatory, in that TLR ligands appear to modulate T cell responses induced by TCR signaling but do not appear to produce functional responses in naïve T cells in the absence of concurrent TCR

stimulation. This may reflect the importance of preventing TLR signals from inducing non-specific T cell activation. In contrast, after having been activated through their TCR, effector and memory T cells appear to maintain responsiveness to TLR ligands in the absence of concurrent TCR-induced signals(Gelman et al. 2004)(Cottalorda et al. 2009).

Functional responses of TLR9-induced co-stimulation in B6 CD4 T cells can be attributed to two main signaling pathways mediated by the adaptor molecule, MyD88. As has been shown in multiple other cell types, stimulation with CpG oligonucleotides (CPG-ODN) induces MyD88-dependent NF-κB activation in CD4 T cells through homotypic death domain interactions with IRAKs. This NF-κB-dependent pathway upregulates expression of the anti-apoptotic molecule Bcl-xL, and thereby promotes activated CD4 cell survival(Gelman et al. 2004). TLR2 stimulation has been found to similarly enhance survival and Bcl-xL expression in activated CD8 T cells(Cottalorda et al. 2006). While NF-κB activation alone appears to be required for CpG-ODN to induce anti-apoptotic signals in T cells, this pathway synergizes with a PI-3-kinase dependent pathway in promoting proliferative responses and IL-2 production(Gelman et al. 2006). In contrast to the death domain interactions by which MyD88 activates NF-κB, MyD88-dependent PI-3 kinase activation involves a tyrosine residue located in the TIR domain. This allows MyD88 to associate with the p85 regulatory subunit of PI-3 kinase and induce Akt phosphorylation in response to TLR stimulation(Gelman et al. 2006). Thus, specific outcomes of TLR-mediated stimulation in T cells may be associated with discrete MyD88 motifs and signaling pathways. It will be of interest to determine whether induction of this PI-3-kinase signaling pathway is specific to TLR9, which is

expressed intracellularly, or are also shared by other TLRs expressed on the cell surface, such as TLR2.

Interestingly, stimulation of CD4 T cells with TLR ligands also results in enhanced expression of secondary co-stimulatory receptors that are generally upregulated following initial T cell activation(Rahman et al. 2009). Certain co-stimulatory receptors, such as CD154, play an important role in allowing CD4 T cells to promote B cell immune responses, which may relate to the finding that MyD88 expression in CD4 T cells is critical in supporting B cell-class switching to IgG production in an *in vivo* model of CpG DNA- induced antibody responses against a T cell dependent antigen(Gelman et al. 2006). Secondary co-stimulatory receptors can also play an important role in supporting sustained T cell responses. For example, signals delivered through OX-40 serve to enhance CD4 T cell effector responses, survival and IL-2 production(Gramaglia et al. 2000). Given that T cell responses induced by these co-stimulatory receptors are quite similar to those that occur following TLR stimulation it is possible that, in addition to the direct TLR-induced signaling pathways discussed above, some of the functional outcomes of TLR stimulation on T cells may reflect secondary consequences of enhanced signaling through these induced co-stimulatory pathways.

The studies described thus far have focused on survival, proliferation and effector cytokine production as readouts of functional TLR responses in T cells. However, in considering other aspects of T cell physiology it has been found that LPS treatment increased adhesion of mouse and human T cells to fibronectin and inhibited chemotaxis(Zanin-Zhorov et al. 2007). Human T cells were also reported to respond

similarly to the endogenous ligand HSP60 through TLR2, though these results could reflect potential contamination of commercially available HSP60 with bacterial TLR2 ligands(Zanin-Zhorov et al. 2006). These results suggest that, in addition to enhancing T cell effector functions, TLR signaling in T cells may also play a role in controlling T cell trafficking. The finding that T cells may potentially respond to endogenous TLR ligands is particularly intriguing since this presents the possibility that DAMPs may act to recruit T cells and support their responses at sites of tissue inflammation and necrosis. These findings also suggest that T cells may respond to a broader range of TLR ligands than has been thus far been considered, since functional outcomes may differ in responses to specific TLR ligands.

CD4+CD25+ Regulatory T cells

The studies discussed thus far indicate that TLR ligands can promote more effective T-cell mediated immunity by acting directly on conventional CD4 and CD8 T cells to enhance their respective effector functions. However, TLR ligands can also influence CD4+CD25+ regulatory T cells (T_{reg}). T_{reg} are known to inhibit T cell effector responses *in vitro,* and are critical in maintaining immune tolerance and preventing the development of autoimmunity *in vivo*(Piccirillo & Shevach 2004). As in the case of effector T cells, TLR ligands indirectly modulate regulatory T cell responses by promoting inflammatory cytokine production in APCs, which can inhibit the suppressive capacity of T_{reg}(Pasare & Ruslan Medzhitov 2003). However, it is now clear that TLR ligands can also act directly on T_{reg}, though there are differences in the reported outcomes of these actions.

The TLR2 ligand, Pam_3CysSK_4, can act directly on mouse T_{reg} through TLR2 to promote their expansion both *in vitro* and *in vivo*, which may relate to the reduced numbers of T_{reg} found in *Myd88-/-* and *Tlr2-/-* mice(Sutmuller et al. 2006). Yet while promoting increased proliferation of T_{reg}, Pam_3CysSK_4 also abrogates their suppressive activity, which correlates with a transient suppression of Foxp3 expression(Liu et al. 2006). It has also been found that direct TLR8 triggering of human T_{reg} similarly prevents their suppressive activity(Peng et al. 2005). It should be noted though that, in contrast to these studies, others have found that signals through TLR2 and TLR5 can enhance the suppressive function of T_{reg} as well as Foxp3 expression(Crellin et al. 2005)(Zanin-Zhorov et al. 2006).

While TLR ligands may influence suppression in co-culture experiments, this potentially reflects the combined result of distinct effects on both conventional T cells and regulatory T cells. Indeed, co-culture suppression assays in which MyD88-deficiency was restricted to either the responder or T_{reg} population showed that CpG DNA can act directly on T_{reg} to block their suppressive activity, but also acts on effector T cells to render then resistant to suppression(LaRosa et al. 2007). Interestingly, once TLR ligands are removed, T_{reg} fully regain their suppressive phenotypes(Liu et al. 2006)(Sutmuller et al. 2006).

Summary

Overall, these findings suggest a model whereby TLR ligands can act on both effector and regulatory T cells to directly promote T-cell mediated immune responses by enhancing T cell effector functions and clonal expansion through increased proliferation,

survival and a transient loss of suppression. These TLR ligands also serve to concurrently expand the T_{reg} population, which regains its suppressive capacity following clearance of the TLR ligands and can then act to regulate the expanded effector T cell population at the end of the immune response.

It is important, however, to consider a number of caveats when interpreting the results of these *in vitro* studies. In order to show that TLR ligands are able to act directly on T cells, these studies depended on using highly purified T cells. The importance of eliminating APCs in these experiments highlights a significant issue: while TLR ligands can act directly on T cells, their indirect effects through APCs generally overshadow their direct effects on T cell activation in *in vitro* cultures. In addition, these studies generally employ high concentrations of TLR ligands that greatly exceed physiological levels. Furthermore, given that TLR signals appear to stimulate different responses in different cell subsets, the overall consequence of TLR ligands on T cells in a mixed culture of cells is less clear. Overall, these *in vitro* studies importantly showed that TLR signals have the ability to directly stimulate T cell responses. However, the relevance of these signals during a physiological immune response *in vivo* remained unclear. In particular, it was not known whether direct TLR signaling contributes to initial T cell activation, effector cell differentiation, proliferation, survival or the differentiation and maintenance of memory cells during an immune response. These unresolved questions formed the basis of my dissertation work.

MyD88 regulates systemic immune responses against pathogens

Studies in mice lacking TLRs, or adapter proteins, such as MyD88, have shown that these receptors play an important role in regulating systemic immune responses. MyD88-deficient mice have greatly increased susceptibility to a number of eukaryotic, prokaryotic and viral pathogens. These include *Toxoplasma gondii*(Scanga et al. 2002), *Mycobacterium tuberculosis*,(Scanga et al. 2004) herpes simplex virus(Tengvall & Harandi 2008), murine cytomegalovirus(Delale et al. 2005), vesicular stomatitis virus (VSV)(Lang et al. 2007), vaccinia virus(Zhu et al. 2007) and lymphocytic choriomeningitis virus (LCMV)(Zhou et al. 2005).

In these infection models, the immune impairments associated with MyD88-deficiency have generally been attributed to the importance of MyD88 in regulating innate immune responses. However, in light of the recent studies demonstrating that TLRs can directly modulate T cell responses, we thought it important to consider whether defective adaptive immune responses in MyD88-deficient mice also reflects a T-cell intrinsic role for MyD88. In this work, we use lymphocytic choriomeningitis virus (LCMV) as a model infection in which to examine the relative contribution of MyD88 in T cells to the overall antiviral T cell response. Using a series of adoptive transfer experiments, we show that while MyD88 plays a significant T cell extrinsic role in promoting T cell responses, it also play an important T cell intrinsic role in regulating T cell expansion during the primary response to acute LCMV infection (Chapter 1). We also use an inducible flox-cre system to inducibly delete MyD88 expression after the initial phase of T cell expansion to show that MyD88 is not required for the differentiation, maintenance and reactivation of memory T cells (Chapter 2).

Table 1. TLRs, their ligands and localization

TLR	Classical ligands	Distribution and cellular localization
TLR1	*Mycobacteria*: tri-acyl peptides	Ubiquitous surface expression in both humans and mice. Heterodimerizes with TLR2
TLR2	*Gram positive bacteria*: peptidoglycan, lipoproteins	Broad surface expression in both humans and mice. Also found in phagolysosomes. Heterodimerizes with TLR1 and TLR6
TLR3	*Viruses*: double-stranded RNA *Synthetic*: polyinosic polycytidylic acid [Poly(I:C)]	Intracellular expression primarily on mDCs in humans and monocytes and mesangial cells in mice
TLR4	*Gram negative bacteria*: LPS (involves coreceptors CD14, LBP and MD-2)	Broad surface expression in both humans and mice. Intracellular expression in gut epithelium.
TLR5	*Bacteria*: flagellin	Broad surface expression in both humans and mice including gut epithelium.
TLR6	*Mycoplasma*: Diacyl lipoproteins	Primarily expressed on the surface of mDCs, mast cells and B cells. Found in both mice and humans. Heterodimerizes with TLR2
TLR7	*Viruses*: single-stranded RNA *Synthetic ligand*: imidazoquinolines	Endosomal expression primarily by pDCs and B cells in humans. Also expressed by mDCs in mice
TLR8	*Viruses*: single-stranded RNA *Synthetic ligand*: imidazoquinolines	Endosomal expression by human myeloid cells, NK cells and T cells. Not reported functional in mice
TLR9	*Viruses and bacteria*: double-stranded DNA *Synthetic*: CpG olidodeoxynucleotides	Endosomal expression primarily on pDCs, B cells and NK cells in humans. Broader tissue expression in mice
TLR10	Unknown	Expressed primarily on the surface of pDCs and B cells in humans. Not found in mice
TLR11	*Toxoplasma gondii*: profilin *Uropathogenic bacteria*	Expressed on the surface of murine uroepithelium. Not described in humans

References: (Hornung et al. 2002)(Ozinsky et al. 2000)(Takeda et al. 2003)(Iwaki et al. 2002)(Aliprantis et al. 1999) (Patole et al. 2005)(Alexopoulou et al. 2001)(Muzio et al. 2000) (Jiang et al. 2000)(Akashi et al. 2000) (Hayashi et al. 2001)(Gewirtz et al. 2001) (Heil et al. 2004)(Jurk et al. 2002) (Hemmi et al. 2000) (Latz et al. 2004) (Yarovinsky et al. 2005)(Zhang et al. 2004)

CHAPTER 2: Materials and Methods

Mice

C57BL/6 (B6), B6.PL-*Thy1*^{a}/CyJ (B6 Thy1.1), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (B6 CD45.1), B6.129-*Tr2*^{tm1Kir}/J, (*Tlr2-/-*), B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ, (*Tlr4-/-*), B6.129S7-*Il1r1*^{tm1Imx}/J, (*IL1r-/-*) and B6.129P2-*Il18r1*^{tm1Aki}/J, (*IL18r-/-*) were purchased from The Jackson Laboratory. *Tlr9-/-* mice were provided by Robert Eisenberg (University of Pennsylvania)(Hemmi et al. 2000). *Myd88-/-* mice were provided by Shizuo Akira (Osaka University)(Adachi et al. 1998). *Myd88-/-* were maintained as a breeding colony in our facility and were crossed with B6 Thy1.1 and B6 CD45.1 to generate Thy1.1 and CD45.1 congenic *Myd88-/-* mice. P14 TCR transgenic mice, which possess CD8 T cells that recognize the gp33 epitope of LCMV(Pircher et al. 1989), were provided by Hao Shen (University of Pennsylvania) and were crossed to generate Thy1.1 congenic *Myd88-/-* P14 mice.

Myd88$^{flox/flox}$ mice have been previously described and were provided by Dr. Anthony DeFranco (UCSF) and backcrossed at least 7 generations onto the C57BL/6 background(Hou et al. 2008). R26R^{YFP} reporter mice on a C57BL/6 background were provided by Jonathan Maltzman (University of Pennsylvania). R26R^{YFP} mice contain an enhanced yellow fluorescent protein (YFP) gene inserted into the *Gt(ROSA)26Sor* locus(Jeong et al. 2004). Expression of YFP is blocked by a loxP-flanked STOP fragment placed between the *Gt(ROSA)26Sor* promoter and the *Smo/EYFP* sequence(Jeong et al. 2004). These strains were intercrossed to generate *Myd88*$^{flox/flox}$ R26R^{YFP} mice, in which

expression of Cre-recombinase allows deletion of MyD88 detectable by the YFP reporter. CreT2 mice on a C57BL/6 background were provided by Jonathan Maltzman (University of Pennsylvania). These mice express a fusion protein of Cre-recombinase fused to a mutant estrogen receptor under transcriptional control of the ubiquitin promoter that is expressed in all reported tissues(Ruzankina et al. 2007). This CreT2 fusion protein is ordinarily restricted to the cytoplasm but can gain access to the nucleus following exposure to 4-hydroxytamoxifen. CreT2 mice were crossed with *Myd88-/-* mice to generate $Myd88^{+/-}$ CreT2 mice. These mice were then crossed with $Myd88^{flox/flox}$ $R26R^{YFP}$ mice to generate $Myd88^{flox/-}$ $R26R^{YFP}$ CreT2 mice and $Myd88^{flox/+}$ $R26R^{YFP}$ CreT2 controls. These mice allow tamoxifen-inducible deletion of MyD88, identified by YFP expression. Since male CreT2 mice may occasionally express low levels of Cre recombinase in germ cells in the absence of tamoxifen treatment (Jonathan Maltzman, unpublished observations) we avoided using breeder mice that expressed both the CreT2 transgene and floxed Myd88 alleles. Thus, our breeding strategy was specifically designed so that the CreT2 transgene was only expressed together with the floxed MyD88 and R26RYFP reporter genes in experimental and but not in breeder mice. $Myd88^{flox/flox}$ mice were also crossed with CD4 Cre mice that express Cre recombinase under the control of the CD4 promoter(Lee et al. 2001) to generate mice with a T cell-specific deletion of MyD88. All colonies were maintained under specific pathogen free conditions in accordance with the protocols of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Mice were genotyped using DNA isolated from tail clippings or ear clippings. Tissues were digested and DNA was purified using either a DNeasy Blood and Tissue Kit

(Qiagen) or a REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturers' instructions. PCR was performed using either PureTaq Ready-to-go PCR beads (GE Healthcare) or the REDExtract-N-Amp Tissue PCR Kit (Sigma) according to the manufacturers' instructions. The following PCR primers were used to genotype *Myd88-/-* mice: Myd88 forward 5' GGT GGT GGT TGT TTC TGA CG 3', MyD88 reverse 5' AAG GCG GGT CCA GAA CCA GG 3', Neo forward 5' CGG GAG CGG CGA TAC CGT AAA GC 3', Neo reverse 5' GCT TGG GTG GAG AGG CTA TTC GG 3'. The following PCR primers were used to genotype *MyD88flox* mice: forward: 5' GTT GTG TGT GTC CGA CCG T 3', reverse: 5' GTC AGA AAC AAC CAC CAC CAT GC 3'. The following PCR primers were used to genotype $R26R^{YFP}$ mice: ROSA1: 5' AAA GTC GCT CTG AGT TGT TAT 3', ROSA2: 5' GCG AAG AGT TTG TCC TCA ACC 3', ROSA3: 5' GGA GCG GGA GAA ATG GAT ATG 3'. The following PCR primers were used to genotype CreT2 mice: hCre forward: 5' TGG GCG GCA TGG TGC AAG TT 3', hCre reverse: 5' CGG TGC TAA CCA GCG TTT TC 3'. Expression of the P14 TCR transgene and CD45 and Thy1 congenic markers was determined by bleeding mice and using antibodies against Vα2 TCR, Vβ8 TCR and the relevant congenic markers to phenotype PBMCs by flow cytometry, as described below.

LCMV infection

Acute LCMV infection was induced by the Armstrong strain of LCMV (LCMV-Arm). LCMV was grown, titered and provided by the labs of Hao Shen (University of Pennsylvania) and E. John Wherry (The Wistar Institute, PA). Virus stocks were diluted

to 4 X 10^5 P.F.U. in sterile PBS and 2 X 10^5 PFU were injected per mouse via intraperitoneal injection. Secondary re-infection was induced by injecting 2 X 10^6 PFU of LCMV Clone 13 (LCMV-CL13) intravenously into mice that had been infected with LCMV-Arm 50-60 days earlier.

***In vivo* tamoxifen treatment**

Tamoxifen (Sigma) was prepared by mixing in 1g/mL of ethanol. This mixture was then diluted to 20mg/mL with corn oil and dissolved by incubating at 37^{o}C for several hours with constant mixing. To induce deletion, Myd88$^{flox/-}$ and Myd88$^{flox/+}$ mice were treated with 200μg tamoxifen/g bodyweight for 5 consecutive days by oral gavage(Park et al. 2008). Mice were bled 5 days following the last day of treatment and successful treatment was confirmed by the presence of YFP+ cells.

Western blotting to verify MyD88 deletion

MyD88$^{flox/-}$ and MyD88$^{flox/+}$ mice were treated with tamoxifen as indicated above. 5 days after the last day of treatment, spleens and lymph nodes were isolated from treated mice and untreated controls and stained for flow cytometry as described below. The YFP+ and YFP- populations were sorted from total splenocytes, CD4 T cells and CD8 T cells. Sorted cells were first lysed at 4°C in lysis buffer composed of 50 mM Tris-HCL (pH 6.8), 0.2% 2-mercaptoethanol, 20% glycerol, 4% SDS, and 0.001% bromophenol blue (Sigma-Aldrich). Cell lysates were clarified by centrifugation at 11,000 g for 10 minutes.

Supernatants were boiled for 5 minutes, separated on a 10% SDS-PAGE at $1x10^6$ cell equivalents/well and blotted onto Hybond ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 30 minutes in 5% milk in TBST at room temperature and then probed with anti-MyD88 antibody (2127, ProSci Inc.) at a 1:500 dilution in 3% milk in TBST overnight at 4°C. Membranes were washed and probed with a horseradish peroxidase–conjugated anti-rabbit secondary antibody at 1:1,000 for 60 minutes at room temperature. Blots were visualized by enhanced chemiluminescence (Roche Diagnostics) according to the manufacturer's protocol. Antibodies were subsequently stripped from membranes using Restore Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed as above with an anti-actin antibody at a 1:5000 dilution.

***In vivo* 5-bromo-2-deoxyuridine (BrdU) administration**

For short term BrdU pulse (24 hours and under) mice were given an intraperitoneal injection of 1mg BrdU (BD Biosciences) every 12 hours. For longer pulses mice were given an initial intraperitoneal injection of 1mg BrdU (BD Biosciences) and were then given a solution of 1mg/mL BrdU (Sigma) and 1mg/mL dextrose (Sigma) in their drinking water. Water bottles were protected from light and changed every 72hrs. To detect BrdU incorporation, splenocytes were isolated from treated mice. Following staining of surface antigens as described below, cells were fixed, permeabilized and stained for BrdU expression using the FITC BrdU flow kit (BD Pharmingen) according to the manufacturer's instructions.

Antibodies, flow cytometry and cell sorting

Single cell suspensions were washed in FACS buffer (PBS + 1% FBS) and stained in polystyrene round bottom tubes or in 96-well round bottom plates for 30 minutes at 4^{o}C. When staining with tetramers, cells were stained for 20 minutes at room temperature. The following antibodies were used: FITC anti-CD44 (IM7), FITC-anti CD69 (H1.2F3), FITC anti-CD45R/B220 (RA3-6132), FITC anti-CD19 (1D3), FITC anti-CD4 (RM4-4), FITC anti-CD4 (GK1.5), FITC anti-CD8 (53.67), FITC anti-CD3e (17A2), FITC anti-CD11b (M1/70), FITC anti-CD11c (N418), FITC-anti Gr1/Ly6G (RB6-8C5), FITC anti-NK1.1 (PK136), FITC anti-γδTCR (Gl-3), FITC anti- Ly67 (Ter119), FITC anti-CD107a (1D4B), FITC anti-CD107b (ABL-93), FITC anti-CD122 (TM-β1), FITC anti-KLRG1 (2F1), FITC anti-Vα2 TCR (B20-1), FITC anti-BrdU, FITC anti-Bcl-xL (7B2.5), PE anti-Annexin V, PE anti 41BB/CD137 (1AH2), PE anti-OX40/CD134 (OX86), PE anti-ICOS (7E.17G9), PE anti-CD40L/CD154 (MR-1), PE anti-PD1 (J43), PE anti-Fas/CD95 (MFL3), PE anti-CTLA4/CD152 (UC10-4F10-11), PE anti-CD25 (PC61) PE anti-CD127 (A7R34), PE anti-Vβ8.1/Vβ8.2 TCR (MR5-2), PE anti-Sca1/Ly-6A/E (E13-161.7), PE anti IL-2 (JES6-5H4), PE anti-granzyme B (GB11), PE anti Bcl-2 (3F11), PerCP anti-CD90.1 (OX7), PerCP-Cy5.5 anti-CD45.1 (A20), PerCP-Cy5.5 anti CD45.2 (104) PerCP-Cy5.5 anti-CD127 (A7R34), PerCP anti-CD25 (PC61), APC anti-CD62L (MEL-14), APC anti-cKit/CD117 (2B8), APC anti-IFNγ (XMG1.2), APC anti-TNFα (MP6-XT22), PE-Cy7 anti-CD62L (MEL-14), PE-Cy7 anti-CD90.2 (53-2.1), PE-Cy7 anti CD45.1 (A20), PE-Cy7 anti IFNγ (XMG1.2), PE-Cy7 anti PD-1 (RMP1-30), Alexa

Fluor® 700 anti-CD4 (L3T4), APC-Alexa Fluor® 750 anti-CD8 (53-6.7), APC-Alexa Fluor® 750 anti-CD45.2 (104), APC-Alexa Fluor® 750 anti CD25 (PC61), Pacific Blue® anti-CD44 (IM7), Pacific Blue® anti-CD8 (53-6.7) and Pacific Blue® anti IFNγ (XMG1.2).

All antibodies were purchased from BD Biosciences, eBioscience or Biolegend except for KLRG-1 (Beckman Coulter), anti-human Granzyme B (Caltag Laboratories), anti-Bcl-xL (Southern Biotech). APC conjugated H2-D^b gp33, H2-D^b np396 and H2-D^b gp276 tetramers were provided by Hao Shen and E. John Wherry.

Flow cytometric analysis was primarily performed on a modified FACS Canto cytometer with an additional Violet laser (Beckton Dickinson). Some analysis was also performed on FACS Calibur and LSR II cytometers (Beckton Dickinson). High speed cell sorting was performed on a FACS Aria (Beckton Dickinson). Flow cytometric data was analyzed using FlowJo software (Treestar Inc.).

Cell isolations for adoptive transfers and analysis

Spleens and lymph nodes were pooled from indicated donor mice and single cell suspensions were prepared by crushing and filtering through 70μM mesh. Red blood cells were lysed using ACK lysing buffer (Lonza) and T cells were isolated using negative selection MACS T cell isolation kits (Miltenyi Biosciences). Enriched T cells were resuspended in HBSS and transferred into recipients via retro-orbital injection. For polyclonal T cell transfer experiments 1 X 10^7 WT or *Myd88-/-* cells were transferred

into B6 Thy1.1 recipients. For P14 cotransfer experiments purified Thy1 congenic WT and *Myd88-/-* T cells were counted and combined at an equal ratio and transferred into B6 CD45.1 congenic recipients. To examine T cell responses at the peak of expansion, day 7 post infection, 2.5 X 10^4 combined P14 cells were transferred. To examine early T cell proliferation, 1 X 10^6 combined CFSE-labeled P14 cells were transferred.

For progressive analysis of T cell differentiation, 5-6 drops of blood were serially collected from infected mice via retro-orbital or sub-mandibular bleeds. Lymphocytes were isolated using Histopaque 1083 (Sigma), resuspended in FACS buffer and stained with antibodies for flow cytometry as indicated above. For terminal analysis mice were sacrificed and spleens and lymph nodes were dissected. In some experiments liver, lung and bone marrow were also collected and a peritoneal lavage was performed. Single cell suspensions were prepared by crushing tissues and filtering through 70μM mesh. Red blood cells were lysed using ACK lysing buffer (Lonza) and in the case of cells from liver, lung, bone marrow and peritoneal lavage, lymphocytes were enriched using Histopaque 1083. Cells were then resuspended in FACS buffer and stained with antibodies for flow cytometry as indicated above.

Generation of mixed bone marrow chimeras

Bone marrow was flushed from femurs and tibias of WT CD45.2 and *Myd88-/-* CD45.1 donor mice using a 20 gauge needle. Red blood cells were lysed using ACK lysing buffer (Lonza) and T cells were depleted using MACS T cell isolation kits (Miltenyi Biosciences). WT and *Myd88-/-* T cell depleted bone marrow was counted and combined

at equal ratio. 1-2 X 10^6 cells were transferred into lethally irradiated (1000 rads) B6 CD45.2, Thy1.1 recipients. In some experiments, ACK lysed bone marrow was stained with PE Sca1, APC cKit and a FITC lineage cocktail (CD4, CD8, CD3ε, γδTCR, CD19, B220, NK1.1, CD11b, CD11c, Gr-1, Ter119) and the hematopoietic stem cell-containing lineage-, Sca1+, cKit+ (LSK) population was sorted. Equal numbers of WT and *Myd88-/-* LSKs were sorted and 2X10^4 combined LSKs were transferred into lethally irradiated (1000 rads) B6 CD45.1 recipients. 12-16 weeks were allowed for immune reconstitution before mice were used for experiments,

***Ex vivo* restimulation and assessment of T cell effector functions**

Spleens were isolated from mice at the indicated time points following infection and single cell suspensions were made as indicated above. LCMV derived peptides were provided by Hao Shen and E. John Wherry and included GP_{33-41}, GP_{61-80}, $NP_{396-404}$ and a pooled peptide mixture comprising $NP_{396-404}$, $NP_{205-212}$, $NP_{166-175}$, $NP_{235-243}$, GP_{33-41}, $GP_{276-286}$, $GP_{118-125}$, GP_{92-101}, and GP_{70-77} that accounts for almost all known LCMV epitopes(Masopust et al. 2007). 3 X 10^6 splenocytes were cultured in round bottom plates with 0.1μg of the indicated LCMV derived peptides in the presence of 0.67μL/mL of Golgistop (containing monensin, BD Biosciences). In experiments to assess degranulation, FITC anti-CD107a and anti-CD107b were also added at the start of culture. Cells were harvested after 5-6 hours and surface stained. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions. Cells were then stained with PE anti-IL2, APC anti-TNFα and PE-Cy7 anti-

IFNγ. For granzyme B analysis cells were stained directly ex vivo without any restimulation.

Assessing *ex vivo* T cell survival

Spleens were isolated from mice at the indicated time points following infection and single cell suspensions were made as indicated above. Splenocytes were either examined immediately *ex vivo* or were cultured overnight in RPMI 1640 with or without 10% FBS. In experiments to examine whether cytokines could rescue cells from apoptosis, media was supplemented with either 100U/mL recombinant mouse IL-2 (R&D Systems) or 1000U/mL Universal Type-I IFN (PBL Biomedical Laboratories) as indicated. ApoStat (FITC-VD-FMK, R&D Systems) was added to cultures for the last 30 minutes of culture as per the manufacturer's instructions to detect total active caspases. After staining of surface marker, cells were resuspended in 200μL of annxein binding buffer with 4μL of annexin V PE (BD Pharmingen) and 7mL of the vital dye 7-AAD to assess apoptosis.

***In vitro* activation-induced cell death (AICD) assay**

Total T cells were MACS purified from spleens and lymph nodes of naïve *Myd88-/-* CD45.2 and congenic WT B6.CD45.1 mice as indicated above. Purified T cells were combined at a 1:1 ratio and cultured with 1μg/mL plate-bound αCD3 and 1μg/mL soluble αCD28 at a density of 3 X 10^6cells/mL/well in a 24-well plate. 100U/mL of IL-2 was added to the cells after 24 hours. Following another 24 hours, cells were harvested

and live cells were isolated using Histopaque 1083 (Sigma). Live cells were re-plated at a density of 1 X 10^6 per well in a 96 well round bottom plate. Cells were either allowed to rest, or were restimulated with either 10μg/mL plate-bound αCD3, 1μg/mL of soluble αFas antibody (BD Biosciences), or 50ng/mL recombinant TNFα (R&D Systems). Cells were harvested at 18, 24 and 48hrs following restimulation. ApoStat (FITC-VD-FMK, R&D Systems) was added to cultures for the last 30 minutes of culture as per the manufacturer's instructions to detect total active caspases. After staining of surface marker, cells were resuspended in 200μL of annxein binding buffer with 4μL of annexin V PE (BD Pharmingen) and 7mL of the vital dye 7-AAD to assess apoptosis. CD4 and CD8 T cells were examined separately during analysis and WT and *Myd88-/-* cells were distinguished on the basis of CD45.1 expression

Assessing the effects of *in vitro* stimulation of T cells with TLRs

Naïve ($CD44^{low}$,$CD62L^{high}$,$CD25^{low}$) CD4 or CD8 T cells were FACS purified from pooled spleens and lymph nodes of WT or *Myd88-/-* mice. Sorted T cells were cultured with 1μg/mL plate-bound αCD3 in the presence or absence of 1μM CpG-ODN (TCCATGACGTTCCTGACGTT) and 1000U/mL murine IFN-α1 (PBL Biomedical Laboratories) as indicated. After 48 hours, culture supernatants were collected to examine levels of secreted IL-2 and IFNγ by ELISA and cells were washed from plates and lysed using lysis buffer composed of 50 mM Tris-HCL (pH 6.8), 0.2% 2-mercaptoethanol, 20% glycerol, 4% SDS, and 0.001% bromophenol blue (Sigma-Aldrich). Cell lysates were separated on a 10% SDS-PAGE at 1x10^6 cell equivalents/well and blotted onto Hybond

ECL nitrocellulose membranes (Amersham Biosciences) as indicated above. Blots were probed with a rabbit anti-Bcl-xL antibody (Cell Signaling) followed by a horseradish peroxidase–conjugated anti-rabbit secondary. Following visualization, antibodies were stripped and blots were reprobed as above with an anti-actin antibody.

Examining naïve T cell responses to Type I IFNs

CD8 T cells were MACs purified from WT or *Myd88-/-* mice as indicated above. To examine proximal signaling in response to stimulation with Type I IFNs, purified T cells were either rested or stimulated with 1μg/mL plate-bound αCD3 for 24hrs. Cells were then culture for 30 minutes in the presence or absence of 10000U/mL of murine IFNα1 or 1000U/mL Universal Type-I IFN (PBL Biomedical Laboratories) as indicated in figures. Cells were then lysed and lysates were separated on a 10% SDS-PAGE at $1x10^6$ cell equivalents/well and blotted onto Hybond ECL nitrocellulose membranes as indicated above. Blots were probed using a rabbit anti-phosphpo-STAT 3 antibody (Cell Signaling) followed by a horseradish peroxidase–conjugated anti-rabbit secondary. After visualization, antibodies were stripped and blots were reprobed as above using a rabbit anti-phospho-STAT 1 antibody (Cell Signaling). Antibodies were once again stripped and blots were reprobed using an anti-actin antibody.

To examine longer-term effects of Type-I IFNs, MACs purified WT or *Myd88-/-* CD8 T cells were cultured for 72hrs with 0.3μg/mL or plate-bound αCD3 and 2.5μg/mL plate-bound αCD28 in the presence or absence of 10-1000U/mL Universal Type-I IFN (PBL Biomedical Laboratories) or 5ng/mL IL-12 (R&D Systems). Following 72hrs of culture,

cells were either stained with Annexin-V and 7AAD to assess viability, or were fixed and permeabilized to examine levels of granzyme B and IFNγ by intracellular staining.

Quantitative PCR for apoptotic markers

Adoptively transferred WT and *Myd88-/-* P14 cells were sorted from recipients 4 days after LCMV infection by FACS on the basis of congenic markers. RNA was purified from the sorted cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was subsequently repurified and concentrated using the RNeasy MinElute Cleanup Kit (Qiagen). RNA concentration and purity was measured on the basis of absorption at 260nm and 280nm using a spectrophotometer. Equal amounts of RNA were converted to cDNA using a RT^2 First Strand Kit (SA Biosciences) and then used in a Mouse Apoptosis PCR Array (stock # PAMM-012, SA Biosciences) according to the manufacturer's instructions. Amplification was performed using an ABI Prism 7000 thermocycler (Applied Biosystems) and analyzed using ABI Prism 7000 SDS software (Applied Biosystems). ΔCt was calculated and normalized to the average ΔCt of the housekeeping genes, actin, Gapdh and Hprt1. Normalized ΔCt of *Myd88-/-* and WT test samples were then compared and expressed as a relative fold difference of *Myd88-/-* P14/ WT P14.

Full genome microarray

Adoptively transferred WT and *Myd88-/-* P14 cells were sorted and pooled from 20 recipients 5 days after LCMV infection by FACS on the basis of congenic markers. RNA was purified from the sorted cells using the RNeasy Mini Kit (Qiagen) and subsequently repurified and concentrated using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. RNA samples were given to the Functional Genomics Core of the Diabetes and Endocrinology Research Center (University of Pennsylvania) for subsequent processing. Functional Genomics Core staff verified RNA integrity using an Agilent Bioanalyzer and performed a two-color Agilent microarray. WT and *Myd88-/-* RNA samples were divided to perform mircoarrays in technical triplicates. Data were filtered and normalized prior to calculation of fold-changes. The significance of fold-changes was assessed by comparing them to the variation in each gene across triplicates using permutation tests. A fold-change of 1.5 or greater was used as a cutoff to generate a list of differentially expressed genes. The gene lists that were over-expressed and under-expressed in *Myd88-/-* relative to WT P14 cells were then processed separately using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, NIH) to generate functional annotation clusters(Dennis et al. 2003). Significance of associations was determined based on the EASE score, a modified Fisher Exact P-value for gene enrichment analysis.

Statistical analysis

A Student's t-test was used to determine the statistical significance of differences between data sets where indicated. A *p* value of less than 0.05 was considered significant.

CHAPTER 3: MyD88 plays a T cell intrinsic role in controlling primary CD8 T cell expansion during acute LCMV infection

Introduction

Lymphocytic choriomeningitis virus (LCMV) is the prototypic murine arenavirus and is a natural mouse pathogen that has been extensively characterized as a viral infection model. LCMV infection of adult mice can result in different outcomes depending on the strain of the virus, the dose and route of infection and the background strain of the host. The Armstrong strain of LCMV induces an acute infection in B6 mice that elicits a dramatic expansion of CD8 T cells that play an important role in viral clearance. A recent study has found that LCMV-specific effector cells divide at least 15 times and comprise over 80% of CD8 T cell pool at the peak of the response, 8 days after infection(Masopust et al. 2007).

LCMV infection has routinely been employed to study adaptive immune responses to viral infection and numerous reagents have been developed that allow the analysis of T cell responses at the single cell level. The LCMV genome encodes a total of 4 proteins and examination of the nucleoprotein and glycoprotein sequences revealed several potential CD8 T cell epitopes in B6 mice. This has resulted in the generation of arrays of LCMV-derived peptides which, when coupled with intracellular cytokine staining, can be used to examine effector cytokine production in response to specific viral epitopes. More recently, MHC class I tetramers have been developed that allow antigen specific CD8 T cells to be enumerated in the absence of a functional readout.

Furthermore, a TCR that recognizes the gp33 epitope of LCMV in the context of H2-D^b has been cloned and used to generate the P14 TCR transgenic mouse(Pircher et al. 1989). Together, these tools make LCMV an ideal model for examining *in vivo* T cell responses to a natural, viral pathogen.

Myd88-/- mice have been shown to develop a greatly reduced frequency of antigen-specific effector CD8 T cells following LCMV infection. This results in reduced cytokine production and cytolytic activity, which is associated with impaired viral clearance of LCMV-WE and high dose LCMV-Armstrong(Zhou et al. 2005). Similarly, the spleens of mice lacking IRAK-4, the signaling kinase downstream of MyD88, have also been found to contain fewer CD8 T cells following LCMV infection resulting in reduced cytolytic activity and impaired viral clearance(Lye et al. 2008). Together, these studies indicate an important role for TLR signaling pathways in controlling T cell responses to LCMV.

In response to LCMV infection, *Myd88*-/- mice produce significantly lower levels of pro-inflammatory cytokines, consistent with the importance of MyD88 in the innate immune response. In particular, plasmacytoid dendritic cells in *Myd88-/-* mice produce lower levels of Type I IFNs which, in turn, have been shown to play an important role in promoting T cell expansion. Antigen presentation of LCMV-derived peptides may also be impaired. Together, these defects in the innate immune response may be expected to contribute greatly to the impaired adaptive immune response to LCMV in *Myd88*-/- mice described earlier. However, in light of the studies discussed in Chapter 1, which demonstrated that MyD88-dependent pathways can regulate T cell responses *in vitro*, I

wanted to examine whether the impaired T cell response also reflects a direct role for MyD88 in T cells during LCMV infection. In the following sections we directly examine the importance of T-cell intrinsic MyD88 expression during naïve T cell differentiation, proliferation and survival during the phase of clonal expansion following acute LCMV infection.

3.1 *Myd88-/-* mice mount greatly reduced T cell responses to LCMV

Infection of C57BL/6 (WT) mice with LCMV-Armstrong induces a dramatic expansion of CD8 T cells peaking 8 days post infection. Consistent with earlier reports we found greatly reduced CD8 T cell numbers in the spleens of *Myd88-/-* mice following LCMV infection (Fig. 3.1A). Notably, the difference in cell numbers was restricted to cells that expressed high levels of CD44, characteristic of antigen experienced cells.

To further characterize these defects we next used available reagents to specifically examine antigen-specific T cells. We found a greatly reduced frequency of antigen-specific IFNγ-producing CD8 T cells in *Myd88-/-* splenocytes following an *ex vivo* restimulation with H-2D^{b} restricted LCMV-derived peptides (Fig 3.1B). Similarly, we found a reduced frequency of IFNγ-producing CD4 T cells in response to stimulation with the I-A^{b}-restricted gp61-80 peptide (Fig 3.1B). While these findings appeared to indicate a reduced frequency of antigen-specific cells it could also potentially reflect a failure of antigen specific cells in *Myd88-/-* mice to differentiate into cytokine-producing effector cells. To distinguish between these possibilities we used MHC class I tetramers to enumerate CD8 T cells specific for known immunodominant LCMV epitopes

independent of their functional responses. We found a greatly reduced percentage (Fig 3.1C) and absolute number (Fig 3.1D) of antigen specific cells in *Myd88-/-* mice.

These findings suggest that the absence of IFNγ producing cells primarily reflects a reduced frequency of LCMV-specific CD8 T cells at the peak of the response. However, it should be noted that the relative difference in the frequency of tetramer-specific cells between WT and *Myd88-/-* mice (Fig 3.1C) appears to be somewhat less than the difference in the frequency of IFNγ producing cells (Fig 3.1B), suggesting that MyD88 may also contribute to the development of T cell effector functions. In addition to their reduced number, we also observed differences in phenotypic receptor expression on LCMV-specific CD8 T cells from *Myd88-/-* mice. In particular, we found significantly elevated levels of the inhibitory receptor PD-1 on *Myd88-/-* CD8 T cells, most notably within the np396-specific population (Fig 3.1E). This complements the results of a recent study that found that concurrent TLR stimulation promoted lower levels of PD-1 expression on CD8 T cells following peptide vaccination(Wong et al. 2009). Notably, we found that these elevated levels of PD-1 appeared to be correlated with a more dramatic reduction in the np396-specific population of T cells in *Myd88-/-* mice than the gp33 or gp276-specific populations, suggesting that MyD88 expression regulates the pattern of immunodominance in the T cell response. Overall, these findings suggest that MyD88 appears to control the frequency, function and phenotype of LCMV-specific T cells following infection.

3.2 The importance of MyD88 in LCMV cannot be attributed to individual TLR pathways

Given that MyD88 is required for signaling through multiple TLR pathways we wished to examine whether the phenotype of *Myd88-/-* mice following LCMV infection could be recapitulated in mice lacking specific MyD88-dependent receptors. As discussed earlier, B6 T cells have been shown to respond directly to ligands for TLR2 and TLR9 *in vitro*. We therefore infected *Tlr2-/-* and *Tlr9-/-* mice to determine whether T cell responses to LCMV are impaired in the absence of these TLR pathways. In contrast to the severely impaired T cell response in *Myd88-/-* mice, we found no significant reduction in the frequency (Fig 3.2A) or total number (Fig 3.2B) of antigen specific CD8 T cells in the spleens of *Tlr2-/-* or *Tlr9-/-* mice following LCMV infection. In fact, *Tlr2-/-* splenocytes showed an increased frequency of CD8 T cells and an increased frequency of tetramer-specific cells within the CD8 T cell population resulting in a significantly greater overall number of tetramer-specific cells than WT mice ($p = 0.02$) (Fig 3.2B). CD8 T cells from *Tlr2-/-* and *Tlr9-/-* mice also produced IFNγ following *ex vivo* restimulation with LCMV-derived peptides suggesting that the absence of these TLR pathways does not impair CD8 T cell expansion or effector differentiation (Fig 3.2C). These findings are supported by a recent study that also found that the phenotype of *Myd88-/-* mice was not recapitulated in mice lacking individual TLR pathways(Jung et al. 2008).

In addition to TLR signaling, MyD88 also plays a role in signaling downstream of the IL-1R family. Cytokines in this family, including IL-1 and IL-18, have been shown to play a role in controlling T cell survival and effector differentiation(Adachi et al. 1998)(Li et

al. 2007)(Denton et al. 2007). We therefore examined T cell responses following LCMV infection in *IL1r-/-* and *IL18r-/-* mice to see whether these pathways, rather than TLRs may account for the importance of MyD88. We found no reduction in the frequency (Fig. 3.2A) or number (Fig 3.2B) of antigen specific CD8 T cells in *IL1r-/-* mice, and while there were significantly fewer tetramer-specific CD8 T cells in the spleens of *IL18r-/-* mice ($p = 0.03$), the CD8 T cell response was far larger than observed in *Myd88-/-* mice. Furthermore, CD8 T cells from *IL1r-/-* and *IL18r-/-* produced IFNγ upon *ex vivo* restimulation (Fig 3.2C) suggesting that these pathways are also dispensable for effector differentiation. Together, these results suggest that the impaired T cell response to LCMV infection in *Myd88-/-* mice cannot be narrowed down to the role of specific MyD88-dependent receptors. In light of this finding, we decided to continue to focus on *Myd88-/-* cells for subsequent studies.

3.3 Adoptively transferred WT T cells expand better than endogenous T cells in *Myd88-/-* hosts

We have showed that MyD88 plays an important role in controlling T cell responses following LCMV infection, but it was unclear whether this reflected a direct or indirect role in T cells. As discussed earlier, the reduced levels of type I IFNs and other pro-inflammatory cytokines in *Myd88-/-* mice during LCMV infection suggest that the MyD88-deficient innate immune compartment may not effectively support T cell expansion and effector differentiation(Jung et al. 2008). However, given that MyD88-dependent pathways can also directly regulate T cell functions, the impaired T cell

response in *Myd88*-/- mice could potentially reflect an intrinsic role for MyD88 in T cells during LCMV infection.

To examine the relative contribution of MyD88 in the innate immune compartment, we transferred naïve WT CD8 T cells were into congenic, *Myd88*-/- recipients, which were then infected with LCMV (Fig 3.3A). We found that adoptively transferred WT CD8 T cells mounted a reduced response to LCMV infection in *Myd88*-/- hosts compared to their response in WT controls, resulting in 2-fold fewer transferred cells 8 days after infection (Fig 3.3B). This corresponded to a significantly reduced frequency (Fig 3.3C) and number (Fig 3.3D) of antigen-specific, IFNγ-producing cells. Notably, there was a greater reduction in the np396-responsive population than in the gp33-responsive population suggesting that MyD88 in the host environment contributes to the pattern of immunodominance in the T cell response.

The reduced frequency of IFNγ producing cells correlated with a reduced frequency of LCMV-specific cells as identified by tetramer staining (Fig 3.3E), indicating reduced expansion rather than a failure to differentiate into IFNγ-producing effectors. Further examination of the phenotype of these tetramer-specific cells revealed that WT T cells expressed higher levels of PD-1 in *Myd88-/-* hosts than in WT controls (Fig 3.3F). This suggests that the elevated levels of PD-1 observed in *Myd88-/-* mice (Fig 3.1F) are primarily associated with the MyD88-deficient environment rather than an intrinsic characteristic of the T cells.

Importantly however, while the donor WT T cells did not expand as greatly in *Myd88-/-* hosts as in WT hosts, they expanded far better than the endogenous *Myd88*-/-

CD8 T cells as indicated by a greater relative percentage of tetramer-specific cells than the endogenous *Myd88-/-* cells (Fig 3.3F). Therefore, while these results support earlier studies suggesting that MyD88 plays an important, T cell-extrinsic role in supporting T cell responses during LCMV infection, the absence of MyD88 in the host environment does not entirely account for the reduced CD8 T cell response in *Myd88*-/- mice. This suggests that the importance of MyD88 during LCMV infection may be attributable to both T cell-extrinsic and T cell-intrinsic components.

3.4 *Myd88-/-* T cells fail to expand effectively when transferred to WT hosts

To more directly examine the potential contribution of MyD88 in T cells during LCMV infection, we performed the reciprocal adoptive transfer of purified CD8 T cells from naïve *Myd88*-/- mice into congenic WT recipients (Fig 3.4A). Despite the provision of a WT APC compartment and pro-inflammatory cytokine milieu, we found that *Myd88*-/- donor CD8 T cells did not expand as effectively as WT CD8 T cells in response to LCMV, resulting in approximately 10-fold fewer donor cells 8 days after infection (Fig 3.4B). While the *Myd88-/-* donor population generated antigen-specific IFNγ-producing effector cells, their frequency (Fig 3.4C) and overall number (Fig 3.4D) were dramatically reduced. This also corresponded to a greatly reduced frequency of antigen specific cells as detected by tetramer staining (Fig 3.4E), suggesting that MyD88 intrinsically regulates LCMV-specific T cell expansion rather than the development of effector functions.

When comparing these findings to our results in Fig. 3.3 it is striking to note that the reduced expansion of *Myd88-/-* CD8 T cells in WT hosts is far more dramatic than the reduced expansion of WT CD8 T cells in *Myd88-/-* hosts following LCMV infection. Together, these reciprocal transfer experiments suggest that the T cell-intrinsic expression of MyD88 is critical in regulating T cell expansion and is an essential consideration in accounting for the reduced T cell responses observed in *Myd88-/-* mice.

3.5 The impaired LCMV response of *Myd88-/-* T cells does not result from a developmental defect in a *Myd88-/-* environment

These adoptive transfer experiments indicate a T cell-intrinsic role for MyD88 during acute LCMV infection. However, it was possible that T cell development in a MyD88-deficient environment could cause persistent functional impairments, resulting in diminished expansion of *Myd88-/-* LCMV-specific CD8 T cells despite transfer into a WT environment. To address this potential issue we sorted the hematopoietic stem cell-containing LSK populations from the bone marrow of *Myd88-/-* and Myd88+/+ littermates, combined them with congenically disparate WT LSKs and transferred them into lethally irradiated recipients to generate WT/KO and WT/WT chimeras (Fig 3.5A). These mixed bone marrow chimeras allowed us to examine the responses of WT and *Myd88-/-* T cells that developed together in the same mouse.

We found that WT and *Myd88-/-* hematopoietic stem cells had repopulated the hematopoietic compartment comparably by 16 weeks post transplant. Thus, the CD8 T cell compartment contained an equivalent proportion of CD45.1 and CD45.2 cells in both

WT/KO and WT/WT uninfected chimeras (Fig 3.5B and C). We found a similar proportion of CD45.1 and CD45.2 cells in the CD4 T cell compartment of uninfected chimeras. Following LCMV infection, however, we found that the *Myd88-/-* fraction of the CD8 T cell compartment did not expand as effectively as the WT compartment, resulting in a significant decrease in the relative proportion of *Myd88-/-* CD8 T cells in WT/KO chimeras (Fig 3.5 B and C). Notably, overall T cell expansion following infection was comparable in WT/KO and WT/WT chimeras (Fig 3.5C), suggesting that the absence of MyD88 in half of the innate immune compartment did not significantly impair the ability of the innate immune system to support T cell responses to LCMV.

The reduced expansion of the *Myd88-/-* T cell compartment WT/KO chimeras corresponded to a lower frequency of LCMV-specific CD8 T cells as identified by tetramers (Fig 3.5D). However, despite their reduced frequency, LCMV-specific *Myd88-/-* CD8 T cells expressed comparable levels of PD-1 as WT cells (Fig 3.5E) and also produced IFNγ in response to restimulation with H2-D^b restricted LCMV derived peptides (Fig 3.5F). These findings indicate, consistent with our earlier results, that MyD88 regulates CD8 T cell expansion but not differentiation into functional effectors. Notably, restimulation with the I-A^b restricted gp61-80 peptide showed a reduced frequency of LCMV-specific CD4 T cells in the *Myd88-/-* compartment of WT/KO chimeras (Fig 3.5F), suggesting that MyD88 intrinsically regulates both CD8 and CD4 T cell expansion following LCMV infection.

3.6 *Myd88-/-* P14 transgenic T cells do not expand as effectively as WT P14 cells

The results thus far show that MyD88 plays a T cell-intrinsic role in regulating T cell expansion in response to LCMV infection. Since these experiments examined naturally occurring LCMV-specific T cell clones in a polyclonal response, it remained possible that T cell population developed differences in its TCR repertoire in the absence of MyD88, which subsequently influenced its response to infection. To circumvent potential repertoire differences in the polyclonal *Myd88*-/- T cell population, we crossed *Myd88*-/- mice with P14-TCR transgenic mice, whose T cells recognize the LCMV-derived gp33-41 epitope. By further crossing these mice onto congenic backgrounds we were able to isolate WT and *Myd88*-/- P14 cells that could be distinguished on the basis of Thy1.1/Thy1/2 disparity. Combining these cells and co-transferring them into congenic WT recipients allowed us to directly compare the responses of a monoclonal population of WT and *Myd88*-/- cells within the same mouse (Fig 3.6A).

The adoptively transferred P14 population expanded dramatically in response to LCMV infection, but there were 10-fold more WT than *Myd88*-/- P14 cells in the spleens of mice at the peak of the response (Fig 3.6B), consistent with the reduced expansion in the polyclonal *Myd88*-/- T cell repertoire. We wanted to confirm that the reduced number of *Myd88*-/- P14 cells in the spleens of infected mice did not reflect their sequestration in other tissues. Although we noted some differences in their relative tissue distributions, the percentage of *Myd88*-/-, relative to WT, P14 cells was also reduced in the peripheral lymph nodes, peritoneum and liver of infected mice, suggesting that their reduced numbers truly reflected reduced expansion rather than differential trafficking.

3.7 *Myd88-/-* P14 T cells develop effector phenotype and functions following LCMV infection.

The ability to clearly distinguish the transferred P14 cells of congenic markers allowed us to examine whether the reduced expansion of the *Myd88-/-* cells was associated with differences in their effector phenotype or functions. We found no differences in the expression of the T cell costimulatory receptors 41-BB, OX-40, ICOS, CD27 or CD40L (Fig 3.7A). Similarly, we found no differences in the expression of inhibitory receptors PD-1, Fas, or CTLA-4 (Fig 3.7B) or the cytokine receptors CD25, CD127 or CD122 (Fig 3.7C). These results suggest that the reduced expansion of *Myd88-/-* P14 cells is not a secondary effect of reduced costimulation or cytokine signaling. We also found that *Myd88-/-* P14 cells upregulated granzyme B expression and produced IFNγ and TNFα comparably to WT P14 cells (Fig 3.7D). Thus, consistent with our earlier results with polyclonal T cell adoptive transfer and mixed bone marrow chimeras, MyD88 appears to intrinsically regulate CD8 T cell expansion following LCMV infection but not differentiation into functional effectors.

3.8 *Myd88-/-* T cells proliferate comparably to WT T cells in response to LCMV but fail to accumulate.

Our results thus far show greatly reduced clonal expansion of *Myd88*-/- CD8 T cell in response to LCMV. T cell numbers at peak expansion reflect the combined effects of proliferation and cell survival, thus we sought to examine which of these processes involved MyD88. At the peak of the response, the majority of the CD8 T cell population

in WT mice is comprised of rapidly cycling effector cells, a large proportion of which incorporate BrdU during a 24 hour labeling period. In contrast, only a small proportion of the CD8 T cells in *Myd88-/-* mice incorporated BrdU, consistent with a reduced frequency of rapidly expanding effector T cells (Fig. 3.8A). This was consistent with the reduced proportion of LCMV-specific effectors in the CD8 T cell population of *Myd88*-/- mice (Fig. 3.1). However, examination of the tetramer-specific cells revealed that the small population of LCMV-specific *Myd88*-/- CD8 T cells incorporated BrdU comparably to WT CD8 T cells (Fig. 3.8B). This suggested that LCMV-specific T cells in *Myd88*-/- mice proliferated at a normal rate, implying that their dramatically lower numbers reflect impaired survival.

To more comprehensively examine cell proliferation, we co-transferred CFSE-labeled WT and *Myd88*-/- P14 cells into congenic hosts to examine early proliferation in response to LCMV infection. No spontaneous proliferation was observed upon transfer, since both WT and *Myd88*-/- P14 cells remained $CFSE^{high}$ in uninfected recipients (Fig. 3.8C). At 72hrs following LCMV infection, both WT and *Myd88*-/- P14 cells proliferated rapidly and underwent multiple rounds of division, diluting CFSE beyond the level of detection by 96hrs, suggesting that WT and *Myd88*-/- P14 cells proliferate comparably in response to LCMV. While division profiles appeared similar, the relative proportion of *Myd88-/-* to WT P14 cells decreased over time, corresponding to reduced accumulation of *Myd88-/-* T cells (Fig 3.8C and 3.8D). The reduced numbers of *Myd88-/-* P14 cells became most apparent by day 4 post infection, at which point proliferation could no longer be resolved by CFSE dilution. As it was possible that *Myd88*-/- T cells proliferated normally for the first 7-8 rounds of division but subsequently stopped proliferating earlier

than WT T cells, we used BrdU to compare the proportion of transferred WT and *Myd88*-/- P14 cells that were actively cycling at later time points. We found that a similar percentage of the adoptively transferred WT and *Myd88*-/- P14 cells incorporated BrdU during a 12hr labeling period on days 4, 5 and 6 following LCMV infection (Fig 3.8E). Thus, the capacity for proliferation between the two populations appeared to be similar throughout the period during which the number of WT P14 cells greatly exceeded the number of *Myd88*-/- P14 cells.

3.9 Antigen specific *Myd88-/-* T cells from LCMV infected mice exhibit increased spontaneous apoptosis.

Reduced accumulation of LCMV-specific *Myd88-/-* T cells despite comparable proliferation indicated reduced survival. To directly examine the survival of transferred *Myd88-/-* and WT P14 cells following LCMV infection, we compared their viability using light scatter properties, annxein V staining and caspase activation. Surprisingly, we observed no significant differences in the viability of WT and *Myd88-/-* P14 cells directly *ex vivo* at any of the time points examined (Fig 3.9A, left panels and data not shown). Since this could potentially reflect the rapid clearance of apoptotic cells *in vivo*, we isolated splenocytes from infected recipients during the period of T cell expansion and cultured them *ex vivo.* Following 20hrs of culture *ex vivo* we found significantly reduced survival of the *Myd88-/-* P14 cell (Fig 3.9A right panels). This reduced survival resulted in a decline in the relative ratio of live *Myd88-/-* to WT P14 cells during the culture period, consistent with the reduced accumulation of *Myd88-/-* P14 cells seen *in vivo.*

Together, these data indicate that the reduced number of *Myd88*-/- T cells following LCMV infection primarily reflects impaired survival, rather than impaired proliferation.

3.10 *Myd88-/-* P14 cells do not exhibit differences in the survival molecules Bcl-xL, Bcl-2 or Bim.

The *in vitro* studies described earlier showed that TLR ligands can act directly on T cells to increase survival through upregulation of the anti-apoptotic protein, Bcl-xL(Gelman et al. 2004)(Cottalorda et al. 2006). We therefore hypothesized that the reduced survival of *Myd88-/-* CD8 T cells following LCMV infection may be associated with reduced levels of Bcl-xL. However, we did not find significant differences in the expression of the Bcl-xL between WT or *Myd88-/-* P14 cells by intracellular staining (fig. 3.10A). Recent reports have also demonstrated that T cell survival and contraction following LCMV infection are regulated by the anti-apoptotic molecule Bcl-2 and the pro-apoptotic BH3 only protein, Bim(Wojciechowski et al. 2006)(Wojciechowski et al. 2007). We therefore also examined the levels of these molecules but did not find significant differences in their expression between WT and *Myd88-/-* P14 cells (fig 3.10A).

Since the resolution offered by intracellular cytokine staining may not be adequate to detect more subtle differences in the level of expression of these apoptotic proteins we also decided to examine their RNA expression using real-time, quantitative PCR. We sorted WT and *Myd88-/-* P14 cells from infected recipients 4 days after infection and examined the expression of multiple apoptosis-related genes using an apoptosis PCR array. Using this assay we found no notable differences between WT and

Myd88-/- P14 cells in the expression of Bcl-2, Bcl-xL or in other pro and anti-apoptotic Bcl-2 family members (Fig. 3.10B). It should be noted however, that the RNA yields from the sorted cells were in the lower end of the range recommended to be used in these PCR arrays, which potentially reduces the sensitivity of the assay.

3.11 WT and *Myd88-/-* T cells are comparably sensitive to Fas induced cell death.

We have already shown that the reduced expansion of *Myd88-/-* T cells following LCMV infection is not observed in mice lacking individual MyD88-dependent TLR or cytokine receptors (Section 3.2). While this could potentially reflect the redundancy of multiple TLR or cytokine pathways during *in vivo* immune responses, we also considered that the importance of MyD88 during LCMV infection might be related to a role in alternative signaling pathways. As discussed earlier, MyD88 has been shown to participate in Fas-induced signaling pathways in macrophages and fibroblasts due to its ability to interact with other death domain containing proteins. It has been shown that, as a result of these homotypic death-domain interactions, the expression of FADD can inhibit signaling through TLRs by sequestering MyD88 away from TLR signaling pathways(Zhande et al. 2007). We therefore hypothesized that the expression of MyD88 and its interaction with FADD may reciprocally inhibit signaling through Fas, thereby potentially rendering *Myd88-/-* T cell hyperesponsive to Fas induced apoptosis. Fas has recently been shown to contribute to the contraction of the CD8 T cell response following LCMV infection, suggesting that Fas-signaling is a potentially relevant pathway of apoptosis in LCMV-specific T cells(Zhou et al. 2002)(L. T. Nguyen et al. 2000)(Weant et al. 2008). TNF-induced

signaling has been shown to play a role in down-regulating the CD8 T cell response following LCMV infection(Nguyen et al. 2000)(Singh & Suresh 2007) and signals downstream of the TNFRs also involve death domain-containing proteins(Hsu et al. 1995)(Micheau & Tschopp 2003), raising the potential for interactions with MyD88. While we had already found that Fas expression was comparable between WT and *Myd88-/-* P14 T cells following infection (fig. 3.7B), this did not address potential differences in signaling downstream of the Fas receptors. We therefore hypothesized that enhanced signaling through Fas or TNF pathways may account for the increased apoptosis of *Myd88-/-* T cells following LCMV infection.

Naïve T cells upregulate Fas expression in response to TCR-induced activation in the presence of IL-2(Refaeli et al. 1998). Following restimulation, these recently activated T cell undergo Fas-mediated apoptosis in a process termed activation induced cell death (AICD) (Van Parijs et al. 1996)(Refaeli et al. 1998). We therefore employed an in vitro AICD assay to directly compare the susceptibility of WT and *Myd88-/-* T cells to Fas-induced cell death. We used a coculture system of congenically marked cells so that WT and *Myd88-/-* T cells would be exposed to identical stimuli throughout the experiment. Using this assay, we found no differences in AICD of WT and *Myd88-/-* CD4 or CD8 T cells following restimulation with high-doses of anti-CD3 antibody, as assessed by both Annexin V staining and the detection of activate caspases (Fig 3.11A). To address the potential caveat that TCR induced AICD was not occurring through Fas, we also stimulated activated cells with anti-Fas antibody or recombinant TNFα to directly induce Fas or TNFR-induced cell death. Consistent with our results with TCR-induced AICD,

we found no differences in apoptosis or caspase activation between WT and *Myd88-/-* cells (Fig 3.11A). These results show that WT and *Myd88-/-* T cells are comparably susceptible to Fas and TNF induced apoptosis, suggesting that enhanced signaling through these pathways does not account for the increased apoptosis of *Myd88-/-* T cells following LCMV infection.

3.12 WT and *Myd88-/-* T cells show comparable responses to Type I IFNs

Our results suggests that the importance of MyD88 in supporting T cell survival during LCMV infection may not be related to its traditional role in TLR or IL1R family cytokine signaling, or to a role in Fas or TNFR signaling. In considering other potential roles for MyD88 in T cells, we noted that the phenotype of *Myd88-/-* CD8 T cells following LCMV infection was strikingly similar to that of T cells lacking the type I IFN receptor. Similarly to *Myd88-/-* P14 cells, in comparison to WT P14 cells, IFNR-/- P14 cells have been found to undergo increased apoptosis during LCMV infection, which results in greatly diminished expansion despite comparable proliferation(Kolumam et al. 2005)(Aichele et al. 2006). Given these similarities, we considered whether MyD88 may be playing a role in IFN-induced signaling pathways in T cells.

As discussed earlier, MyD88-dependent signals have been shown to play an important role in the induction of Type-I IFN production in APCs through the recruitment of IRF1, 5 and 7(Takaoka et al. 2005)(Honda et al. 2004). However, a role of MyD88 downstream of signaling through IFN-IR is less clear. A recent study found that IFNα treatment enhanced human B cell responses to TLR9 stimulation through

upregulation of MyD88 expression(Giordani et al. 2009). We therefore considered whether an element of the IFN-I induced survival program in LCMV-specific T cells occurred as a result of increased signaling through a MyD88-dependent signaling pathway. To test this possibility we examined whether IFNα, in analogy to B cells, promotes enhanced signaling through MyD88-dependent TLR pathways in T cells. As discussed earlier, treatment of TCR-activated CD4 and CD8 T cells with ligands for TLR2 and TLR9 promotes enhanced survival associated with Bcl-xL upregulation(Gelman et al. 2004)(Cottalorda et al. 2006). Using Bcl-xL upregulation as a readout for T cell responses to TLR stimulation, we found that IFNα did not appreciably enhance MyD88-dependent signaling in T cells (Fig 3.12A).

While it did not appear that IFNα enhanced MyD88-dependent, TLR-induced signaling in T cells, we considered the reciprocal possibility that MyD88 may play a role in supporting IFNR-induced signals. TLR stimulation has been shown to induce STAT1 phosphorylation in macrophages through a MyD88-dependent mechanism(Toshchakov et al. 2002)(Rhee et al. 2003). However, we found that *Myd88-/-* and WT T cells comparably phosphorylated STAT1 in response to stimulation with IFNα, suggesting that MyD88 is not required for STAT1 phosphorylation in T cells (Fig 3.12B). Since the pro-survival effects of IFN-induced signaling in T cells have been attributed to STAT3 signaling(Tanabe et al. 2005) we also examined this pathway and found that *Myd88-/-* T cells similarly did not have a defect in STAT3 phosphorylation following IFNα stimulation (Fig 3.12B).

These experiments suggested that *Myd88-/-* T cells did not have a defect in inducing proximal signals in response to IFNα, but did not rule out a potential role for MyD88 in regulating IFN-induced signals further downstream. To address this issue, we compared longer term responses of WT and *Myd88-/-* T cells to IFN-stimulation. Consistent with earlier reports(Curtsinger et al. 2005), we found that IFN-I and IL-12 promoted increased survival and granzyme B upregulation during *in vitro* CD8 T cell activation and we found no reduction in these responses in *Myd88-/-* T cells (Fig. 3.12C). While these results suggested that MyD88 was not required for type I IFN signaling in T cells, we also considered the possibility that MyD88 may play a role in IFN-signaling specifically in LCMV-specific T cells. We therefore compared the effects of type I IFNs on adoptively co-transferred WT and *Myd88-/-* P14 cells isolated from LCMV infected recipients. Consistent with its role in promoting survival, we found that treatment with type I IFNs was able to rescue *Myd88-/-* P14 T cells from the spontaneous cell death that we had observed following ex vivo culture (Fig. 3.12D). Together, these results suggest that MyD88 is not required for IFN-induced signals in T cells, indicating that reduced responses to type I IFNs do not account for the reduced survival of *Myd88-/-* T cells following LCMV infection.

3.13 Genome-wide microarray suggests differences in the expression of lipid phosphates and calcium signaling molecules in *Myd88-/-* P14 cells following LCMV infection

Since our studies had failed to identify a specific mechanism accounting for the reduced survival of *Myd88-/-* T cells, we decided to perform a full-genome microarray, theorizing that differentially expressed genes in *Myd88-/-* cells may provide insight into the role of MyD88 during LCMV infection. We therefore sorted adoptively cotransferred WT and *Myd88-/-* P14 cells from LCMV infected recipients, isolated RNA and performed an Agilent microarray to examine genome-wide differences in gene expression. We hoped to examine cells early in the response, reasoning that differences in the expression of relevant genes would precede differences in survival, however day 5 post infection was the earliest time point at which adoptively transferred P14 cells had expanded enough to allow us to sort sufficient cells for analysis.

Overall, we noted that gene expression between WT and *Myd88-/-* P14 cells was fairly similar. We found a total of 116 genes that were overexpressed in *Myd88-/-* relative to WT P14 cells and 118 genes that were underexpressed with a fold change of 1.5 or greater. Functional annotation clustering analysis of these genes using the NIH DAVID database (Huang et al. 2009)(Dennis et al. 2003) identified 11 functionally related groups with an enrichment score of 1.0 or greater. Consistent with our earlier findings, we did not observe significant differences in genes related to TLR expression, Bcl-2 family members or type I IFN signaling. Amongst genes that were over-expressed in *Myd88-/-* cells we observed an enrichment of lipid phosphatases involved in phosphotidylinositol signaling (Fig 3.13A). Amongst genes that were underexpressed we observed enrichment of genes involved in calcium signaling (Fig 3.13B) and in amino acid metabolism (Fig 3.13C). We also noted reduced expression of Myd88 and associated inflammatory genes (Fig 3.13D).

Overall, these microarray results support our earlier experiments, suggesting that MyD88 may be acting outside of its traditional role in TLR signaling during LCMV infection. Specifically, MyD88 may be contributing to calcium signaling pathways or amino acid metabolism, raising the possibility that defects in these pathways may account for the reduced survival of *Myd88-/-* T cells during LCMV infection. However, a number of caveats must be considered when interpreting these data. Firstly, while microarrays were performed in triplicate, they involved only a single sample of WT P14 RNA and *Myd88-/-* P14 RNA from 20 pooled infected recipients, making statistical significance difficult to assess. In addition, since cells were examined day 5 post infection, at which point differences in apoptosis and accumulation had already manifested, it is possible that these differences in gene expression represent consequences, rather than causes of the reduced survival of *Myd88-/-* cells. Furthermore, since there is considerable cellular heterogeneity in the LCMV response, it is possible that the *Myd88-/-* P14 cells that are present at this point in the response have already been selected for survival, eliminating the cells that are most susceptible to apoptosis from the analysis. Lastly, since this comparison did not include naïve WT and *Myd88-/-* P14 cells from uninfected animals, it is unclear which of these differentially expressed genes reflect basal differences in *Myd88-/-* T cells versus those that develop during LCMV infection.

Summary

Acute LCMV infection ordinarily elicits a robust expansion of virus-specific CD8 T cells, which play an important role in viral clearance. This expansion is critically

dependent on the presence of MyD88. The results in this chapter support a T cell-extrinsic role for MyD88 in regulating T cell responses, as evidenced by the reduced expansion and a shift in the pattern of immunodominance during the response of WT CD8 T cells to LCMV infection in a *Myd88-/-* host (Section 3.3). This likely reflects reduced production of type I IFNs and other pro-inflammatory cytokines by *Myd88-/-* APCs(Jung et al. 2008), which have been shown to be important in regulating T cell expansion. Importantly, however, our results using polyclonal and TCR transgenic adoptive transfers and mixed bone marrow chimeras show that MyD88 also plays a critical, T cell-intrinsic role in supporting the accumulation of antigen specific CD8 T cells during the response to acute LCMV infection.

Earlier studies have shown that MyD88-dependent signals delivered through TLR stimulation can directly regulate CD4 and CD8 T cell responses in vitro. While our results in this chapter extend these findings to show that MyD88-dependent signals in T cells are important during a physiological immune response to an infection in vivo, there are some notable differences between our findings and those suggested by earlier in vitro studies. Costimulation of naïve mouse T cells through TLR2 and TLR9 was shown to augment proliferation, survival and the production of effector cytokines and granzyme B. In contrast to these studies, our results indicate that MyD88-dependent signals are not required for proliferation or the acquisition of effector functions. Instead, the importance of MyD88 appears to be limited to a critical role in controlling the survival of effector T cells, thereby supporting their sustained expansion and accumulation.

Another notable finding in this chapter is that the dramatically reduced accumulation of *Myd88-/-* effector cells was not recapitulated by the absence of signals through TLR2, TLR9 or IL-1R family members. Thus, the upstream mediators of MyD88-dependent signals in T cells during LCMV infection may not be the TLR that have been suggested by in vitro studies. Furthermore, while TLR-induced survival pathways have been associated with the upregulated of Bcl-xL in vitro, we did not observe notable differences in Bcl-xL expression between WT and *Myd88-/-* T cells following LCMV infection. In addition, the increased apoptosis of *Myd88-/-* T cells following LCMV infection was not associated with an increased sensitivity to Fas mediated apoptosis or reduced responsiveness to type I IFNs. Differences in global gene expression instead indicate increased lipid phosphotase activity and reduced calcium signaling and amino acid metabolism in *Myd88-/-* T cells.

In conclusion, the findings in this chapter clearly demonstrate a previously unappreciated, T cell intrinsic role for MyD88 in regulating T cell expansion during and in vivo immune response to LCMV infection. While MyD88 expression in CD8 T cells during LCMV infection appears to be dispensable for initial activation, proliferation, and differentiation effector T cells, it is required for their survival and accumulation. The identity of the upstream stimuli and downstream signals that link MyD88 to T cell survival remain unclear and raise the possibility that MyD88 may function in an as yet undefined fashion outside of its traditional role in TLR signaling during LCMV infection.

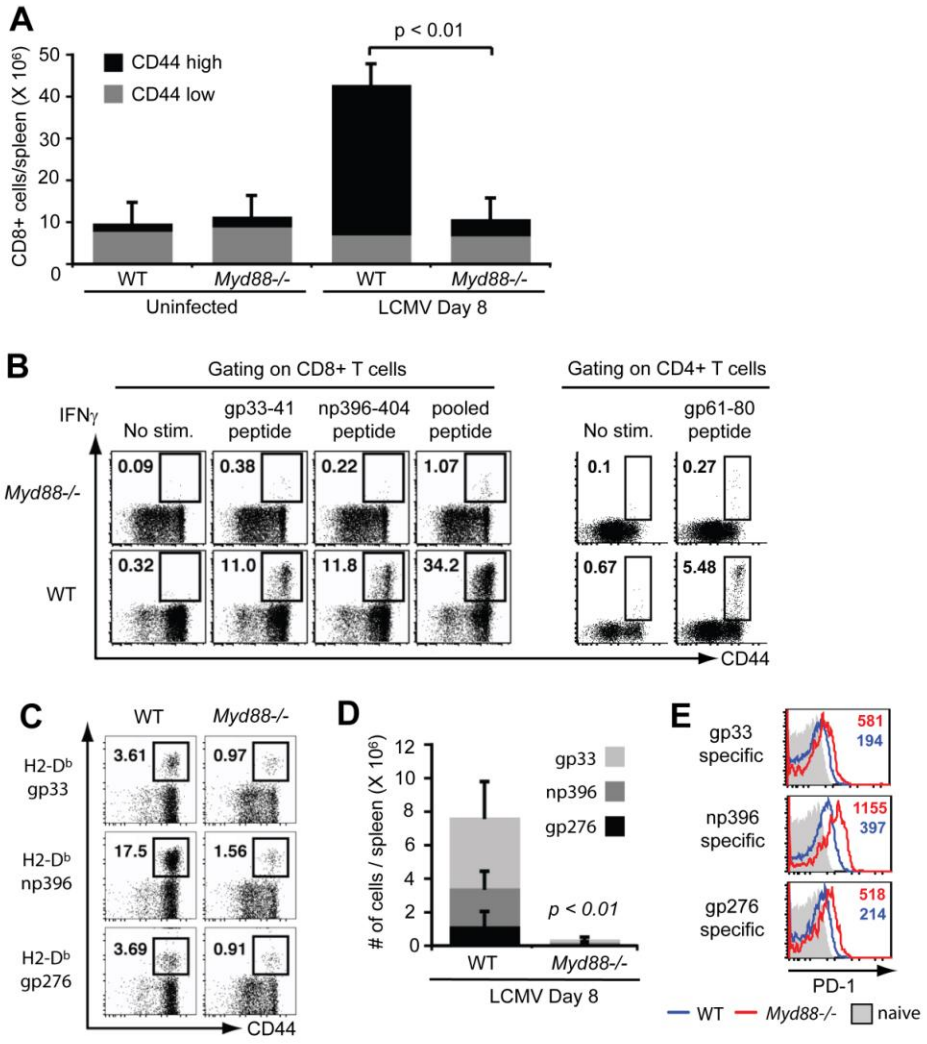


Figure 3.1. *Myd88-/-* mice mount greatly reduced T cell responses to LCMV. WT and *Myd88*-/- B6 mice were infected with LCMV-Arm and splenocytes were purified 8 days after infection. (A) CD8 T cells were enumerated using antibodies against CD8a and CD44. Data represent mean +/- S.D. for at least 10 mice. (B) Splenocytes were restimulated *ex vivo* with the indicated LCMV-derived peptides and antigen-specific IFNγ production was assessed. (C-D) Gated CD8 T cells were analyzed with the indicated tetramers to determine the frequency (C) and number (D) of LCMV-specific CD8 T cells. Data represent mean +/- S.D of at least 10 mice. (E) PD-1 expression on tetramer-specific cells in WT and *Myd88-/-* mice was compared.

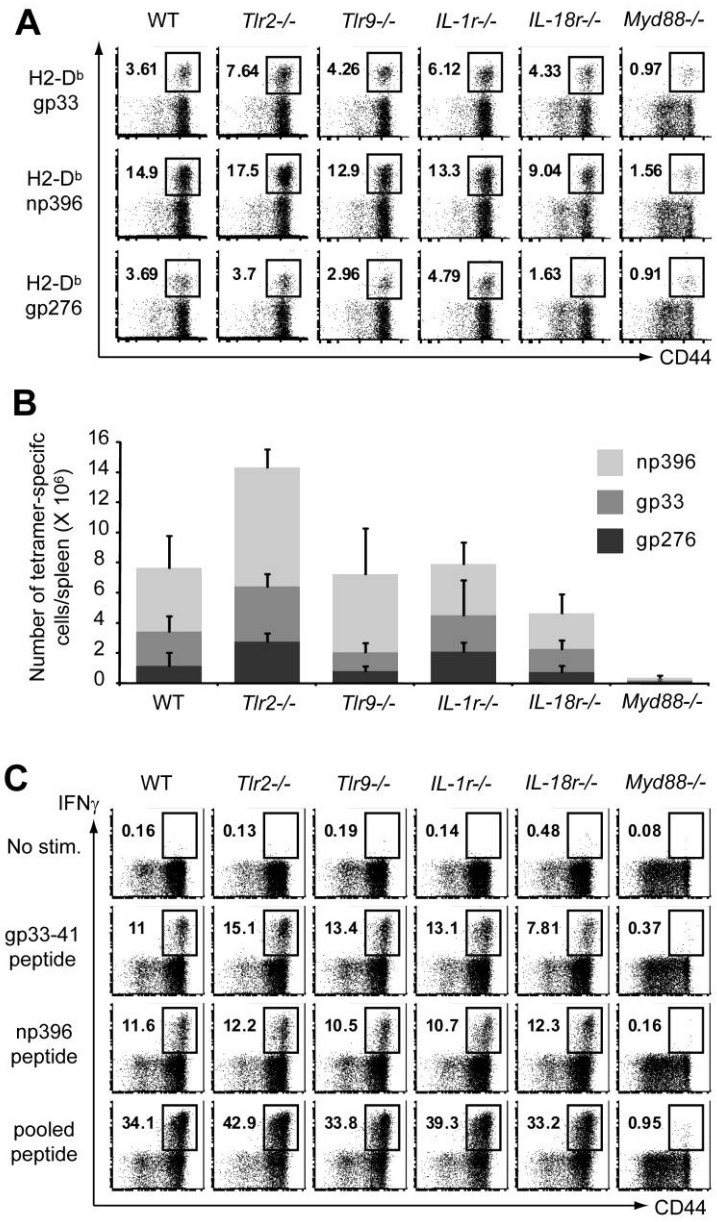


Figure 3.2. The phenotype of *Myd88*-/- mice following LCMV infection is not recapitulated by *Tlr2*-/-, *Tlr9*-/-, *IL-1r*-/- or *IL-18r*-/- mice. The indicated mice were infected with LCMV-Arm and splenocytes were purified 8 days after infection. (A-B) Gated CD8 T cells were analyzed with the indicated tetramers to determine the frequency (A) and number (B) of LCMV-specific CD8 T cells. Data represent mean +/- S.D of at least 5 mice. Splenocytes were restimulated *ex vivo* with the indicated peptides and CD8 T cell IFNγ production was assessed.

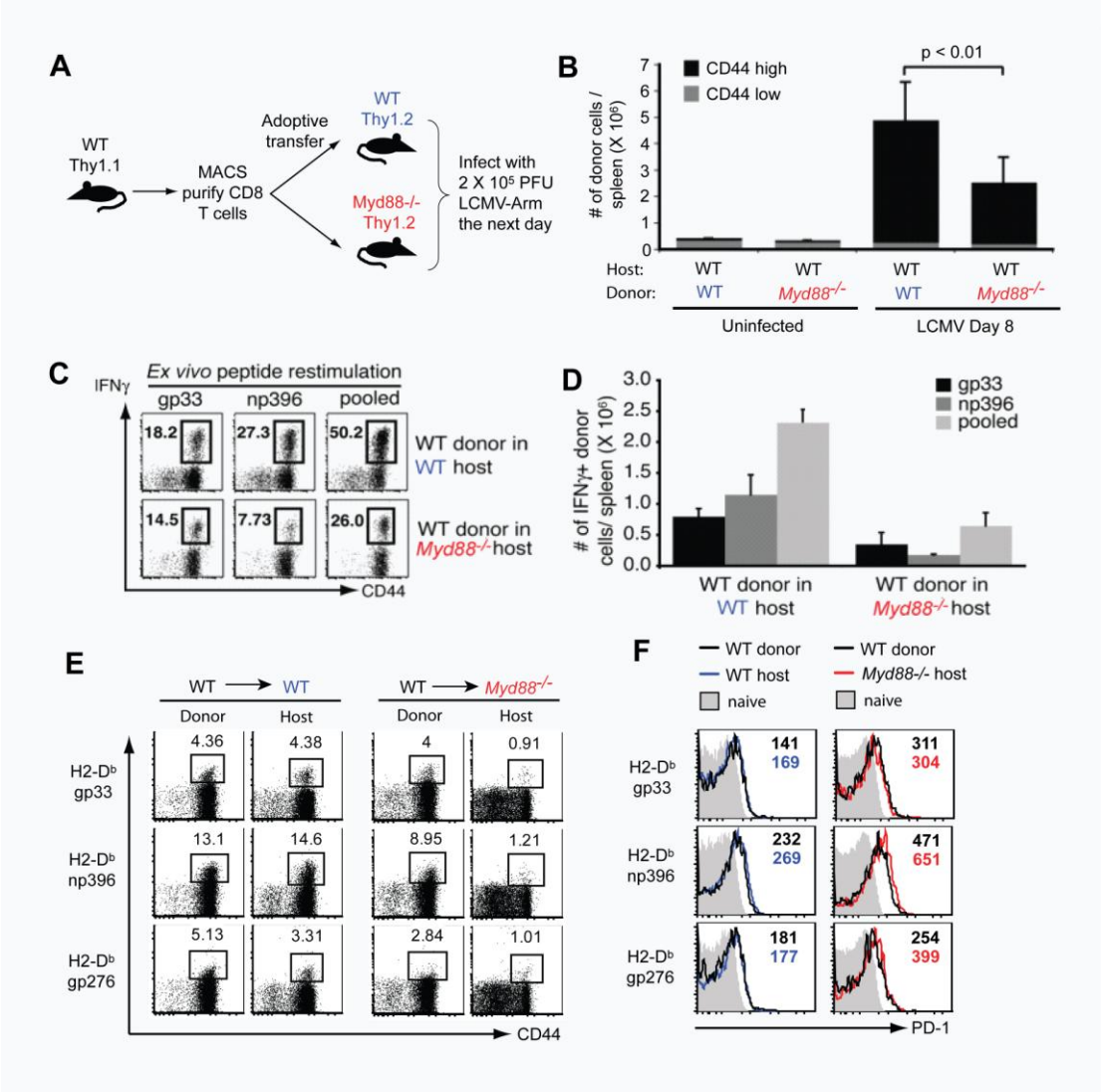


Figure 3.3. WT CD8 T cells are able to expand and develop effector functions in response to LCMV in *Myd88-/-* hosts. (A) Experimental design. Splenocytes were examined 8 days after infection. (B) The number of donor cells per spleen was enumerated. Data represent mean +/- SD of at least 5 mice. (C-D) Splenocytes were restimulated with the indicated LCMV-derived peptides and the frequency (C) and number (D) of IFNγ-producing donor cells was determined. $p < 0.05$ for all values relative to corresponding WT controls. (E) The frequency of tetramer-specific cells in the donor and host CD8 populations was compared. (F) PD-1 expression was examined on donor and host tetramer+ CD8 T cells. Data are representative of at least 5 mice.

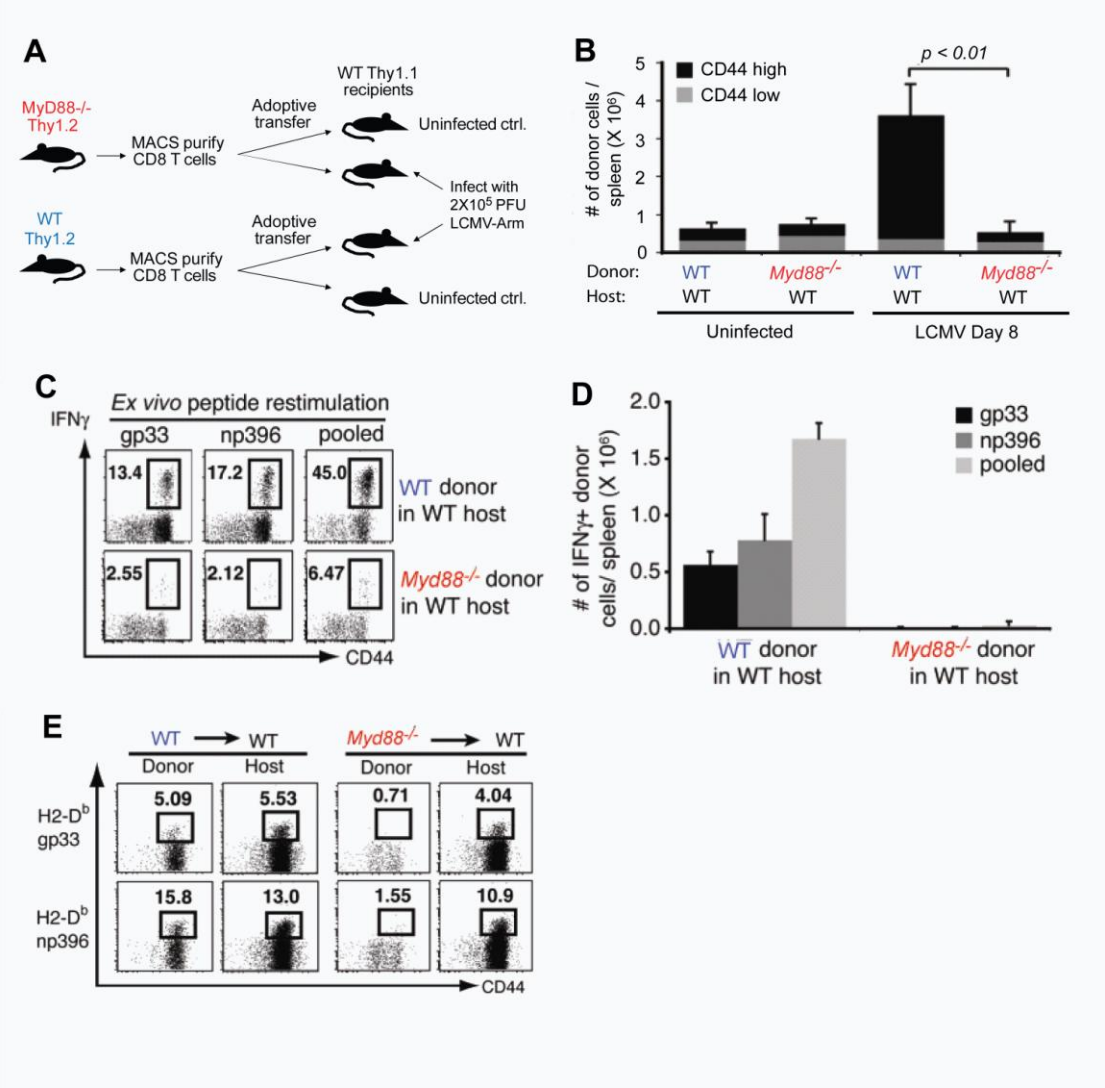


Figure 3.4. *Myd88-/-* CD8 T cells do not effectively expand in response to LCMV infection in WT hosts. (A) Experimental design. Splenocytes were examined 8 days after infection. (B) The number of adoptively transferred cells per spleen was enumerated. Data represent mean +/- SD of 5 mice. (C and D) Splenocytes were restimulated with the indicated LCMV-derived peptides and the frequency (C) and number (D) of IFNγ-producing donor cells was determined. $p < 0.01$ for all values relative to corresponding WT controls. (E) The frequency of tetramer-specific cells in the donor and host CD8 T cell populations was compared. Data are representative of 5 mice per group.

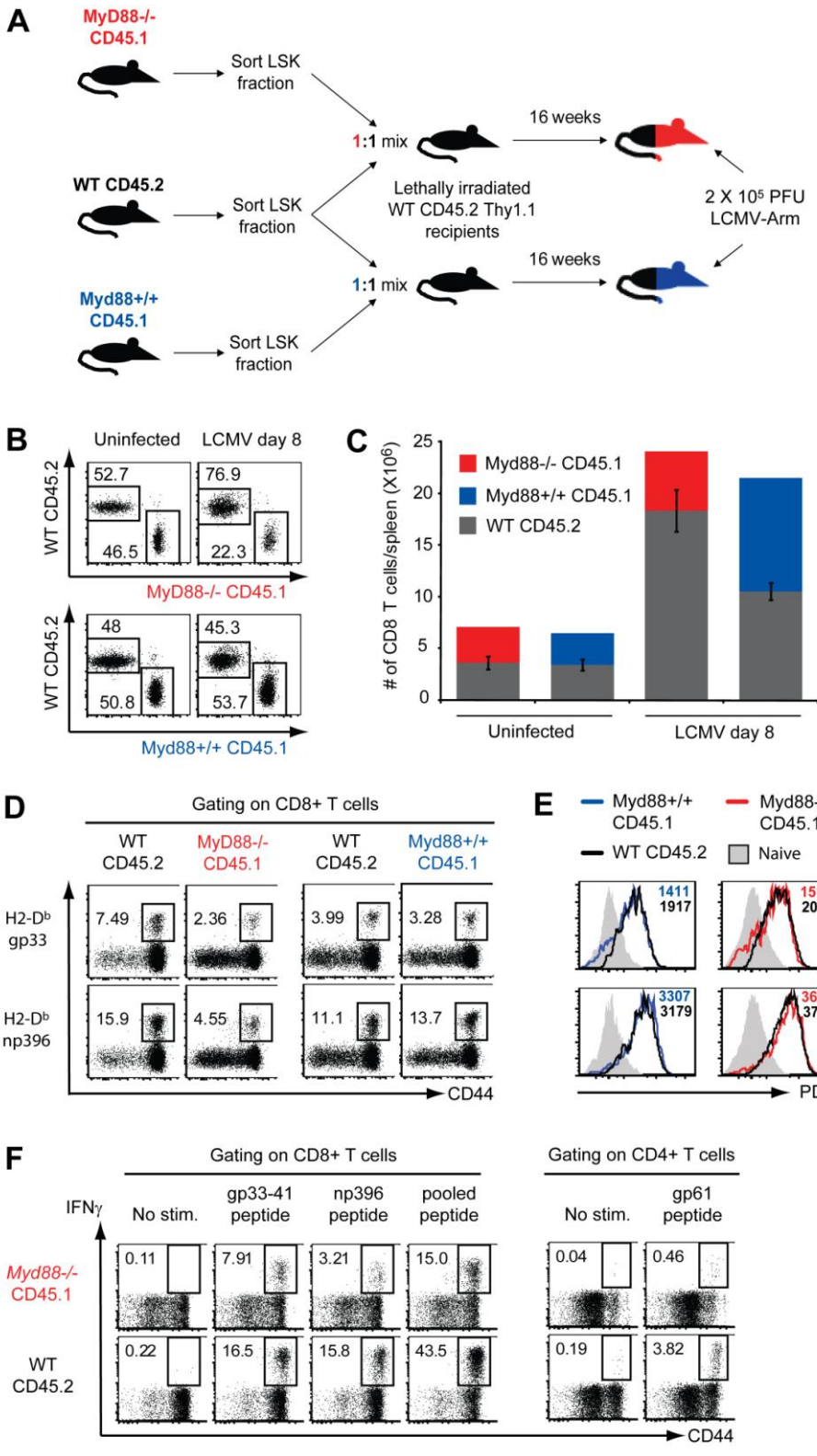

A
MyD88-/- CD45.1
Sort LSK fraction
WT CD45.2
Sort LSK fraction
Myd88+/+ CD45.1
Sort LSK fraction
1:1 mix
1:1 mix
Lethally irradiated WT CD45.2 Thy1.1 recipients
16 weeks
16 weeks
2 X 10⁵ PFU LCMV-Arm
B
Uninfected
LCMV day 8
WT CD45.2
52.7
46.5
76.9
22.3
MyD88-/- CD45.1
48
50.8
45.3
53.7
Myd88+/+ CD45.1
C
# of CD8 T cells/spleen (X10⁶)
Myd88-/- CD45.1
Myd88+/+ CD45.1
WT CD45.2
Uninfected
LCMV day 8
D
Gating on CD8+ T cells
WT CD45.2
MyD88-/- CD45.1
WT CD45.2
Myd88+/+ CD45.1
H2-Db gp33
7.49
2.36
3.99
3.28
H2-Db np396
15.9
4.55
11.1
13.7
CD44
E
Myd88+/+ CD45.1
Myd88-/- CD45.1
WT CD45.2
Naive
1411
1917
1512
2087
3307
3179
3648
3788
PD-1
F
Gating on CD8+ T cells
Gating on CD4+ T cells
IFNγ
No stim.
gp33-41 peptide
np396 peptide
pooled peptide
No stim.
gp61 peptide
Myd88-/- CD45.1
0.11
7.91
3.21
15.0
0.04
0.46
WT CD45.2
0.22
16.5
15.8
43.5
0.19
3.82
CD44

Figure 3.5. ***Myd88*-/- T cell defects following LCMV infection do not arise from development in a MyD88-deficient environment.** (A) Experimental design. Splenocytes were examined 8 days after infection. (B-C) The ratio (B) and number (C) of CD45.1+ and CD45.2+ CD8 T cells was compared in infected and uninfected chimeras. Data show mean +/- SD of 5 mice per group. (D) Tetramers were used to compare the frequency of LCMV-specific CD8 T cells within the CD45.1+ and CD45.2+ populations in the same mouse.(E) PD-1 expression on the tetramer+ CD8 T cells within the CD45.1+ and CD45.2+ in the same mouse was compared. Data are representative of 5 mice and differences in MFI were not significant. (F) Splenocytes were restimulated with the indicated peptides to examine LCMV-specific IFNγ production by WT and *Myd88*-/- CD8 T cells in the same mouse. Data are representative of 5 mice and $p < 0.01$ for all stimulation conditions.

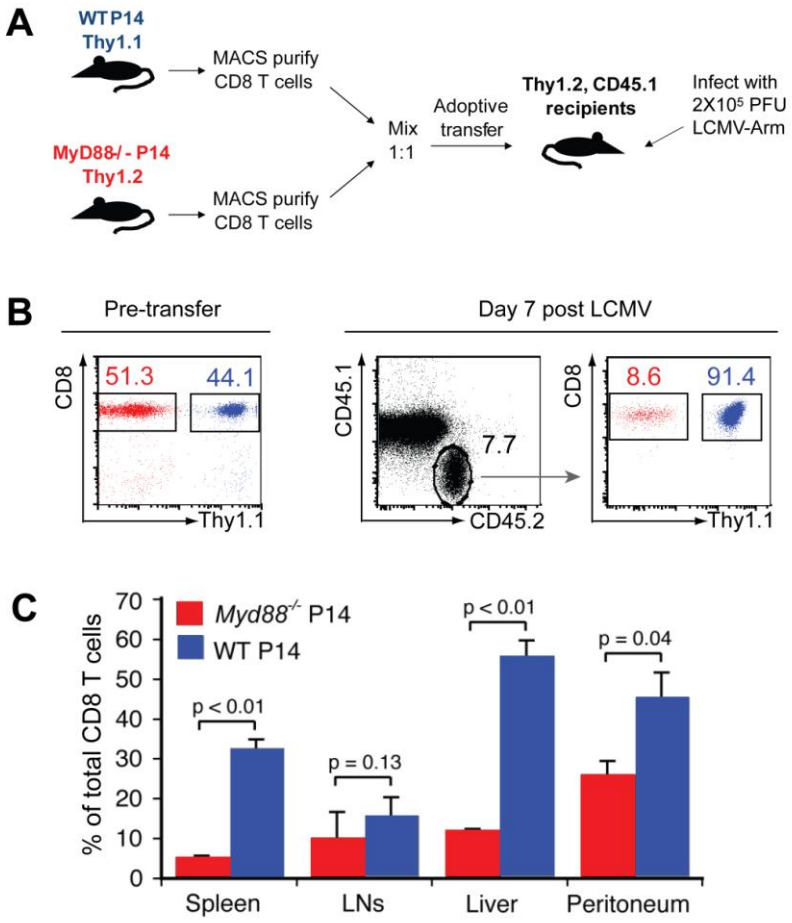


Figure 3.6. *Myd88*-/- P14 TCR transgenic T cells do not expand effectively in response to LCMV infection. (A) Experimental design. (B) The relative proportion of donor *Myd88*-/- P14 to WT P14 cells was compared on the basis of congenic Thy1.1 expression before and after LCMV infection. (C) The frequency of *Myd88*-/- P14 and WT P14 cells within the CD8 T cell population isolated from the indicated tissue compartment was determined using CD45.2 and Thy1.1 congenic marker. Data show mean +/- SD of 5 recipient mice.

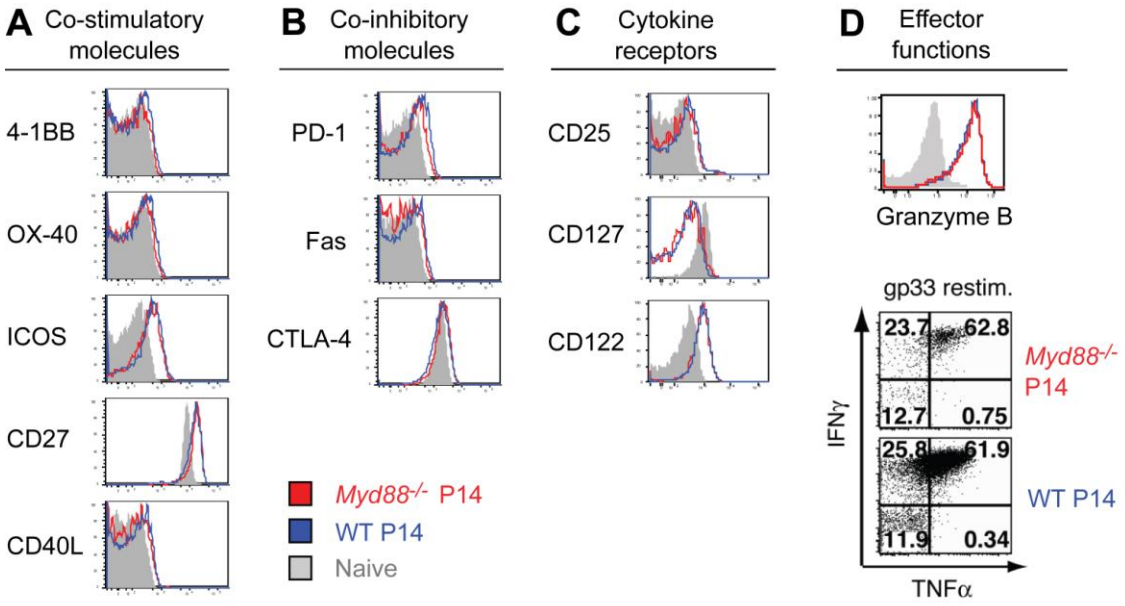


Figure 3.7. *Myd88*-/- P14 TCR transgenic T cells develop a normal effector phenotype and effector functions following LCMV infection. Naïve *Myd88*-/- P14 and WT P14 cells were co-transferred as in Fig. 3.6 and donor derived cells were examined in the spleens of recipients 7 days after LCMV infection. (A-C) The expression of the indicated phenotypic markers was. (D) Granzyme B levels were compared on *Myd88*-/- P14 and WT P14 cells in the same mouse by intracellular staining directly ex vivo. Splenocytes were restimulated with gp33-41 peptide and IFNγ and TNFα production by *Myd88*-/- P14 and WT P14 cells in the same mouse were compared by intracellular staining. All data are representative of 5 mice. No statistically significant differences in MFI were found.

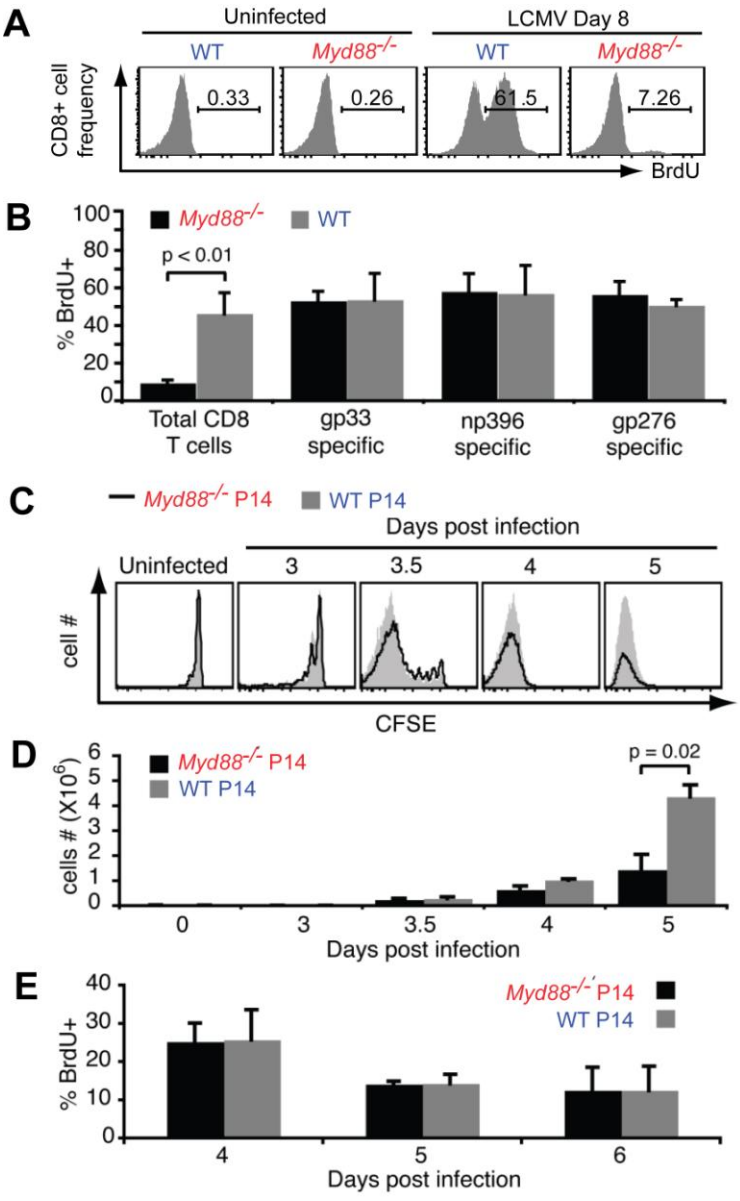


Figure 3.8. *Myd88*-/- and WT CD8 T cells proliferate comparably following LCMV infection. (A-B) WT and *Myd88*-/- mice were infected with LCMV and given a 24hr BrdU-pulse prior to sacrifice on day 8 post infection. BrdU incorporation by (A) overall CD8 T cells and (B) gated tetramer-specific cells was compared. Data show mean +/- SD of 4 mice. (C and D) CFSE-labeled congenic WT and *Myd88*-/- P14 cells were co-transferred into WT recipients which were infected with LCMV 18hrs later. The division profiles (C) and numbers (D) of WT and *Myd88*-/- P14 cells were compared in the spleens at the indicated time points. Data show mean +/- SD of 5 mice at each time point. (E) WT and *Myd88*-/- P14 cells were co-transferred as in (C). Following a 12hr BrdU pulse, recipients were sacrificed at the indicated time points and BrdU incorporation by donor WT and *Myd88*-/- P14 cells was compared. Data show mean +/- SD of 4 mice at each time point.

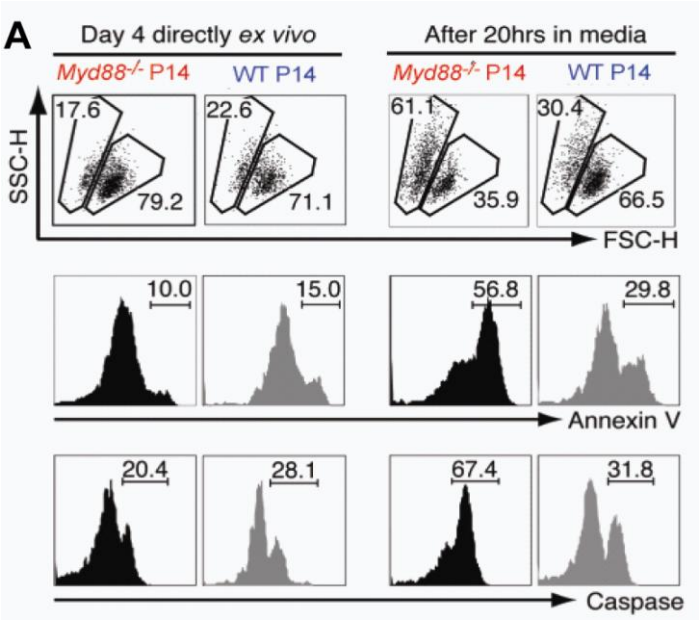


Figure 3.9. *Myd88-/-* CD8 cells exhibit decreased viability following LCMV infection. Naïve WT and congenic *Myd88-/-* P14 cells were co-transferred into WT recipients as in Figure 3.8C. Splenocytes were isolated 4 days following infection and the viability of gated WT and *Myd88-/-* P14 cells was assessed by scatter properties, annexin V staining and the detection of total active caspases. Cells were examined either immediately *ex vivo* (left panels) or following a 20hr culture in the absence of exogenous peptides or cytokines (right panels). Data are representative of 5 mice. $p < 0.01$ for the percentage of live *Myd88-/-* P14 cells relative to WT P14 cells as determined by scatter properties following 20hrs in culture.

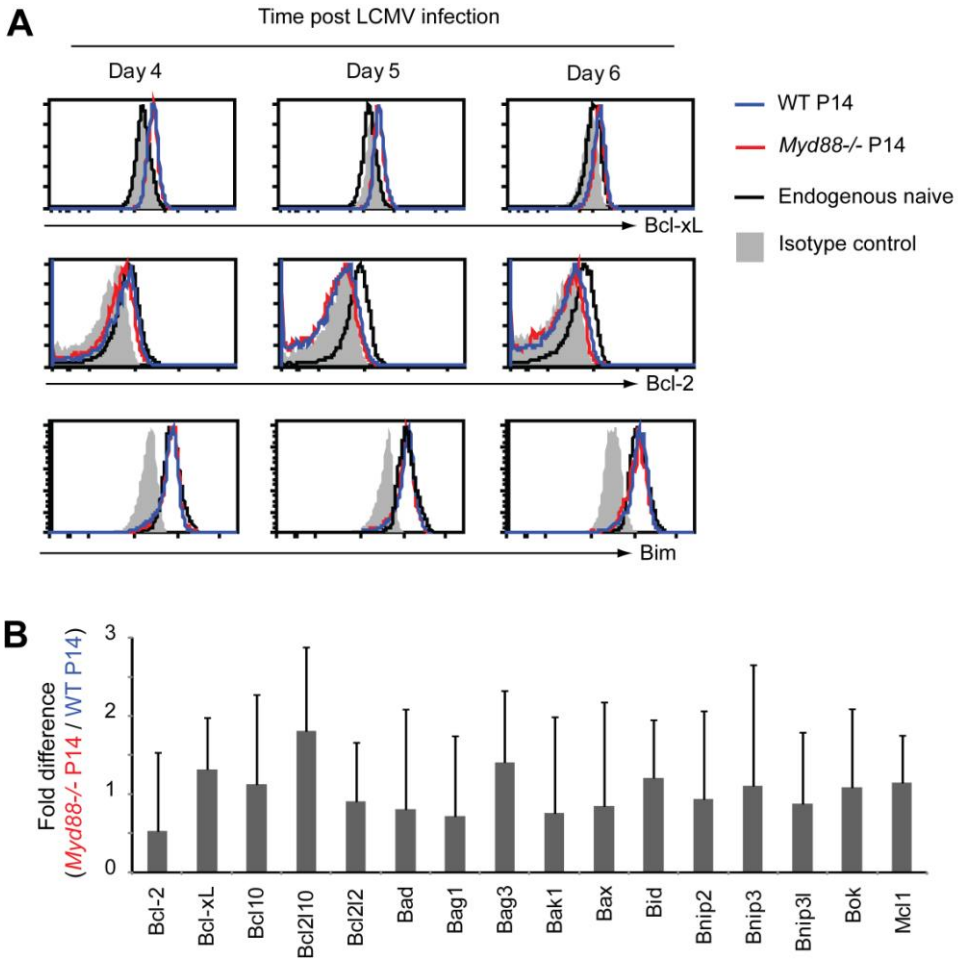


Figure 3.10. *Myd88-/-* and WT P14 cells do not exhibit differences in the expression of Bcl2-family members following LCMV infection. Naïve *Myd88-/-* and congenic WT P14 cells were cotransferred into WT recipients as in Figure 3.6. (A) Splenocytes were isolated at the indicated time point post infection and the expression of Bcl-xL, Bcl-2 and Bim was determined on gated *Myd88-/-* and WT P14 cells by intracellular staining. (C) *Myd88-/-* and WT P14 cells were FACS-purified and pooled from 20 recipients 4 days post LCMV infection. RNA was purified and the expression of the indicated Bcl2 family members was determined using an apoptosis PCR microarray. Data represent mean + SD of two independent experiments.

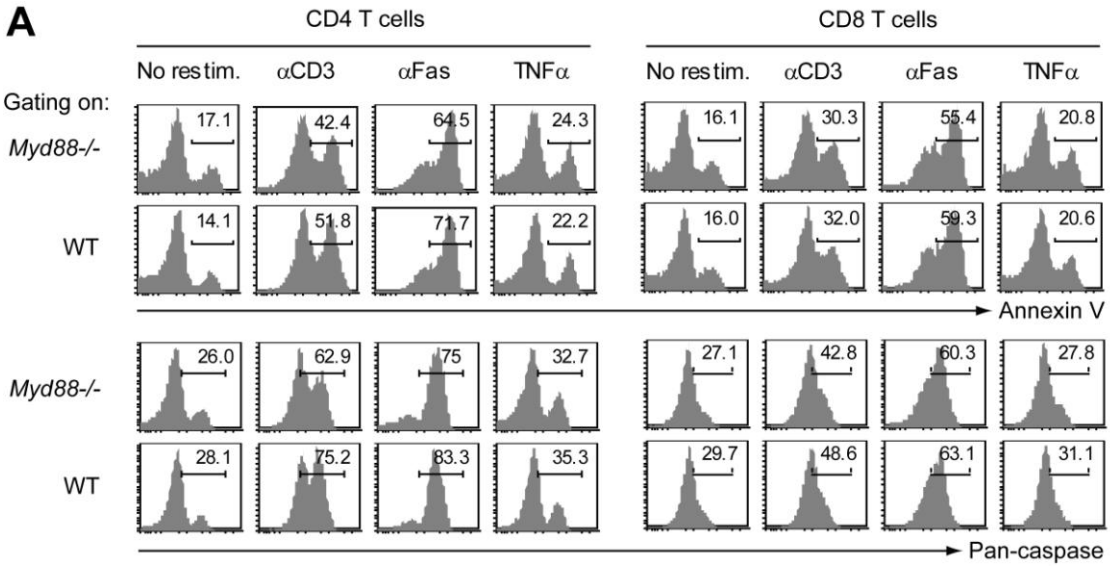


Figure 3.11. *Myd88-/-* and WT T cell are comparably sensitive to Fas and TNF-induced apoptosis. Total T cells were MACS purified from the spleens and lymph nodes of naïve *Myd88-/-* and CD45.1 congenic WT mice and co-cultured at a 1:1 ratio. Following primary activation by plate-bound αCD3 and soluble αCD28 in the presence of IL-2, live cells were isolated and re-cultured under the indicated stimulation conditions. (A) After 24hrs, viability on gated *Myd88-/-* and WT CD4 and CD8 T cells was assessed by the detection of Annexin V and total active caspases. Data are representative of triplicate treatment groups in 2 independent experiments.

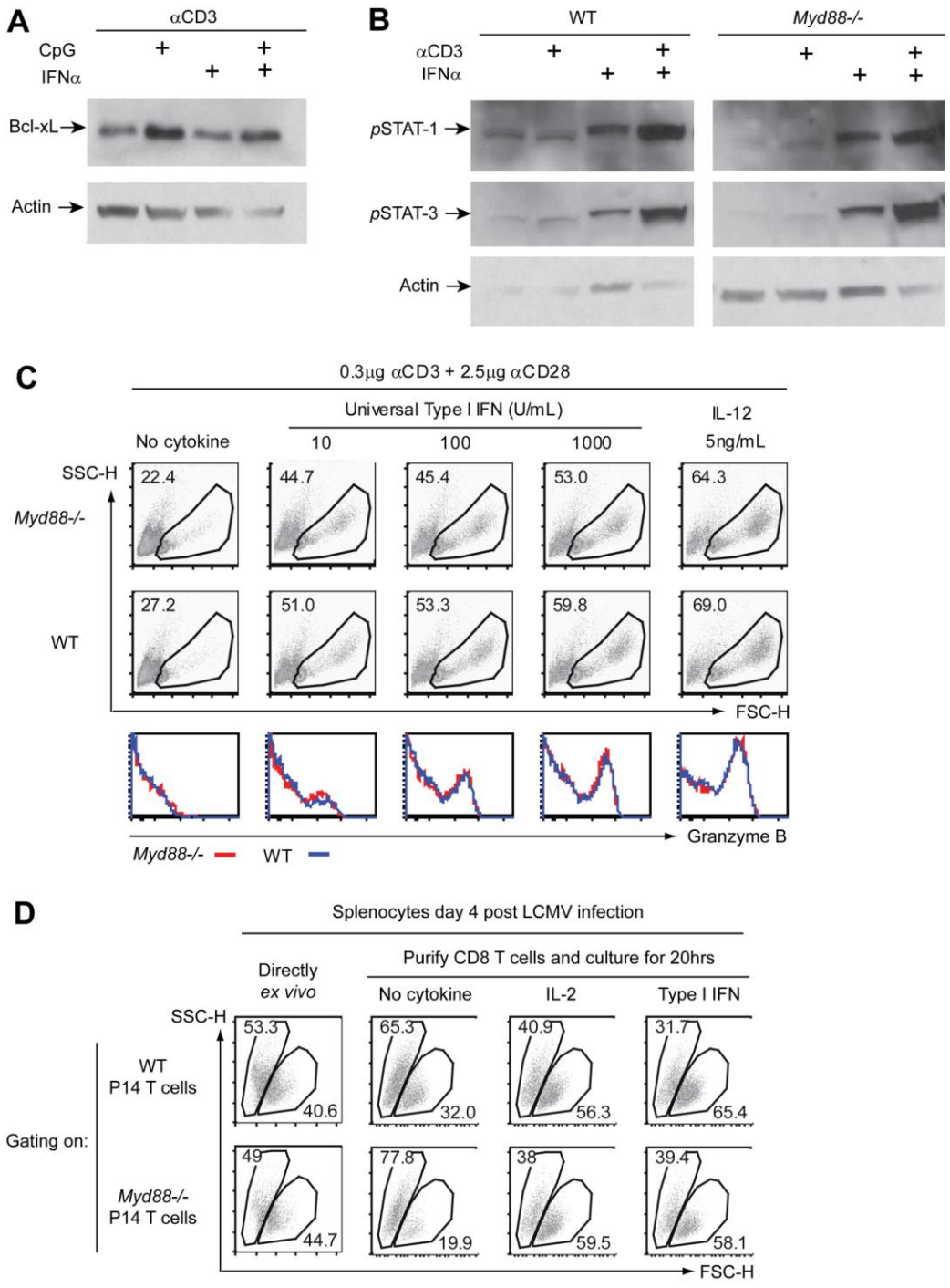
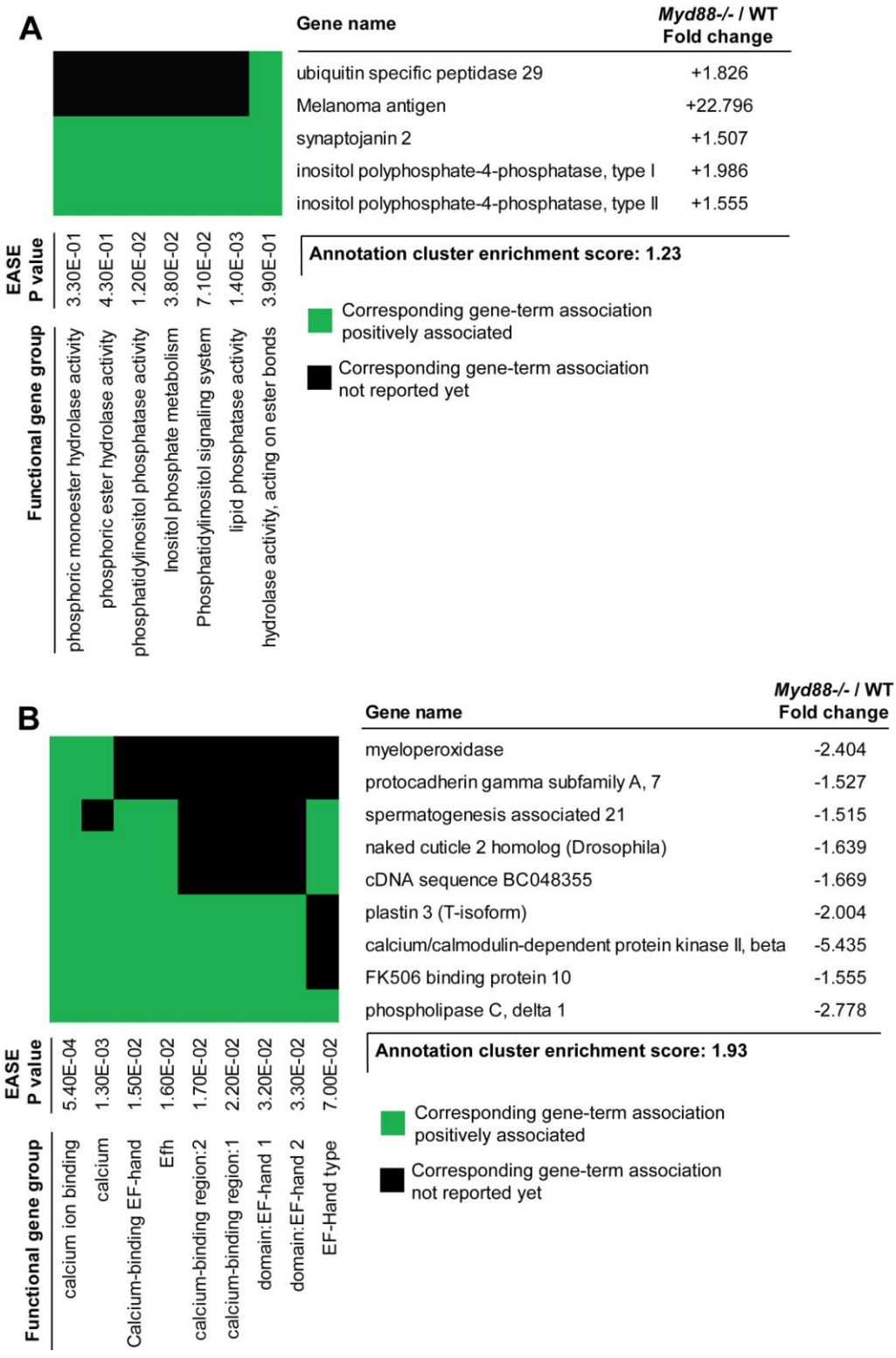


Figure 3.12. MyD88 expression is not required for T cell responses to Type I IFNs. (A) WT CD8 T cells were MACS purified from the spleens and LNs of naïve mice in the presence of plate-bound aCD3 and soluble CpG and recombinant mouse IFNα as indicated. After 48hrs, Bcl-

xL expression was determined by Western blotting. (B) CD8 T cells were MACS purified from the spleens and LNs of naïve WT and *Myd88-/-* mice, cultured for 24 hours in the presence or absence of plate-bound αCD3 and then stimulated with recombinant mouse IFNα for 30 minutes as indicated. The expression of phospho-STAT 1 and phospho-STAT 3 was determined by Western blotting. (C) CD8 T cells were MACS purified from the spleens and LNs of naïve congenic WT and *Myd88-/-* mice and co-cultured at a 1:1 ratio under the indicated conditions. Viability of gated WT and *Myd88-/-* cells was determined by scatter properties (top panels), or cells were fixed and granzyme B expression on gated WT and *Myd88-/-* CD8 T cells was compared following intracellular staining (lower panels). Data are representative of triplicate treatment groups in 2 independent experiments. (D) Naïve congenic WT and *Myd88-/-* P14 cells were transferred into WT recipients as in Figure 3.9. Splenocytes were isolated 4 days after LCMV infection and viability of gated WT and *Myd88-/-* P14 cells was determined based on scatter properties either directly *ex vivo* or following 20hrs of culture under the indicated conditions. Data are representative of triplicate treatment groups in 2 independent experiments.


A
Gene name
Myd88-/- / WT Fold change
ubiquitin specific peptidase 29 +1.826
Melanoma antigen +22.796
synaptojanin 2 +1.507
inositol polyphosphate-4-phosphatase, type I +1.986
inositol polyphosphate-4-phosphatase, type II +1.555
Annotation cluster enrichment score: 1.23
Corresponding gene-term association positively associated
Corresponding gene-term association not reported yet
EASE P value
Functional gene group
3.30E-01 phosphoric monoester hydrolase activity
4.30E-01 phosphoric ester hydrolase activity
1.20E-02 phosphatidylinositol phosphatase activity
3.80E-02 Inositol phosphate metabolism
7.10E-02 Phosphatidylinositol signaling system
1.40E-03 lipid phosphatase activity
3.90E-01 hydrolase activity, acting on ester bonds
B
Gene name
Myd88-/- / WT Fold change
myeloperoxidase -2.404
protocadherin gamma subfamily A, 7 -1.527
spermatogenesis associated 21 -1.515
naked cuticle 2 homolog (Drosophila) -1.639
cDNA sequence BC048355 -1.669
plastin 3 (T-isoform) -2.004
calcium/calmodulin-dependent protein kinase II, beta -5.435
FK506 binding protein 10 -1.555
phospholipase C, delta 1 -2.778
Annotation cluster enrichment score: 1.93
Corresponding gene-term association positively associated
Corresponding gene-term association not reported yet
EASE P value
Functional gene group
5.40E-04 calcium ion binding
1.30E-03 calcium
1.50E-02 Calcium-binding EF-hand
1.60E-02 Efh
1.70E-02 calcium-binding region:2
2.20E-02 calcium-binding region:1
3.20E-02 domain:EF-hand 1
3.30E-02 domain:EF-hand 2
7.00E-02 EF-Hand type

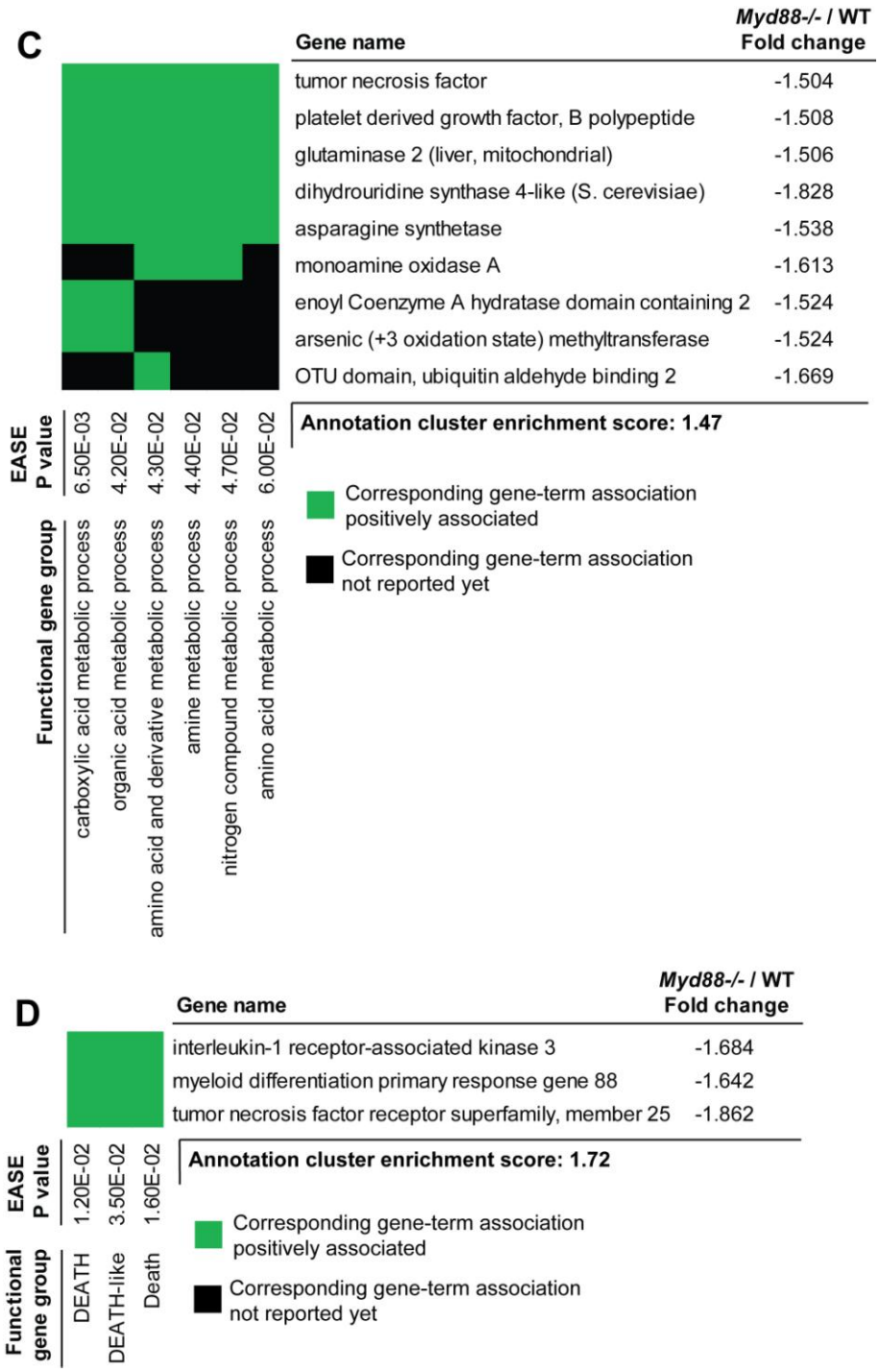


Figure 3.13. *Myd88-/-* P14 cells express higher levels of lipid phosphotase genes and lower levels of genes involved in calcium signaling and amino acid metabolism. Naïve *Myd88-/-* and congenic WT P14 cells were cotransferred into WT recipients as in Figure 3.6. *Myd88-/-* and WT

P14 cells were FACS-purified and pooled from 20 recipients 5 days post LCMV infection. RNA was purified and the gene expression was determined by a full-genome Agilent microarray. Differentially expressed genes with a fold change of 1.5 or greater were grouped into functionally-related clusters using the NIH DAVID software. The 4 clusters with the highest enrichment scores are shown. Data represent results from a single experiment in which pooled RNA samples were divided to perform triplicate microarrays.

CHAPTER 4: MyD88 is not required for the differentiation, maintenance and reactivation of memory T cells following LCMV infection

Introduction

The initial phase of rapid CD8 T cell expansion and differentiation into function effectors during acute LCMV infection is followed by a period of contraction during which 90-95% of T cells undergo apoptosis. The remaining LCMV-specific T cells persist as a stable memory population. These memory T cells are maintained through slow homeostatic turnover that is independent of antigen, and instead relies on signals from the cytokines IL-7 and IL-15(Surh & Sprent 2008). Following subsequent encounter with the same antigen, these memory T cells rapidly undergo expansion and differentiation into secondary effectors. Recent work suggests that the costimulation through TNFR family members plays an important role in maintaining the functional phenotype of memory T cells(Allam et al. 2009). Thus, the signals involved in the regulation of the memory T cell population are distinct from those involved in the regulation of naïve or effector cells.

Our results in Chapter 3 showed that MyD88 plays an intrinsic role in controlling the survival and expansion of T cells during the initial phase of antigen-driven proliferation following LCMV infection. As discussed earlier, it has been found that MyD88-dependent signaling through direct TLR costimulation can promote T cell differentiation into memory cells. Furthermore, memory T cells have been found to

express higher TLR levels and possess greater sensitivity to stimulation with TLR ligands. A recent study has shown that TLR2 ligands can act directly on memory CD8 T in the absence of specific antigen to promote proliferation and cytokine production(Cottalorda et al. 2009). These findings raise the possibility that MyD88-dependent signals may play a role in regulating memory T cells generated in response to LCMV infection. We therefore examined whether the defective expansion of LCMV-specific *Myd88-/-* T cells was also associated with additional defects in their contraction and differentiation into memory cells.

4.1 *Myd88-/-* P14 cells generate a reduced frequency of memory cells following LCMV infection

As we showed earlier, adoptively transferred *Myd88-/-* P14 cells expand considerably less than WT P14 cells resulting in significantly fewer cells in the spleens of recipient mice at the peak of the response, and also a similarly reduced number of memory cells 35 days post infection (Figs. 3.6 and 4.1A). While their initial expansion was reduced, serial bleeds showed that *Myd88-/-* P14 cells contracted with comparable kinetics to WT P14 cells (Fig 4.1B).

The LCMV-specific effector T cell population contains considerable cellular heterogeneity at the peak of the response. In particular, a small subset of effector T cells expresses high levels of the IL-7Rα and low levels of killer cell lectin-like receptor G1 (KLRG1). In comparison to the majority of effector cells, which possess a $KLRG1^{high}IL7R\alpha^{low}$ phenotype, these $KLRG1^{low}IL7R\alpha^{high}$ cells have a considerably

greater potential of developing into long-lived memory cells. Thus, these KLRG1^{low}IL7Rα^{high} cells have been termed memory precursor effector cells (MPECs), while the KLRG1^{high}IL7Rα^{low} cells are primarily short lived effector cells (SLECs). We found that the population of *Myd88-/-* P14 cells contained a comparable proportion of SLECs and MPECs as WT P14 cells at the peak of the response, suggesting that the *Myd88-/-* and WT effector T cell pools contained similar memory potential (Fig 4.1C). Additionally, the majority of the *Myd88-/-* P14 cells that were present 35 days post infection exhibited the KLRG1^{low}IL7Rα^{high} phenotype of memory cells.

Thus, while these data show that *Myd88-/-* CD8 T cells generate fewer memory cells in response to acute LCMV infection, the phenotypic heterogeneity of the *Myd88-/-* effector population, its kinetics of contraction and the similar relative proportions of *Myd88-/-* and WT P14 cells at day 7 and day 35 post infection suggest that the reduced number of *Myd88-/-* memory cells may simply reflect their reduced initial expansion.

4.2 T cells in *Myd88ΔT* mice do not expand effectively in response to acute LCMV infection

It is well established that the magnitude of the initial clonal burst significantly influences the size of the memory T cell compartment(S. Hou et al. 1994). Therefore, in order to more specifically examine whether MyD88 plays a role in the generation and maintenance of memory cells, independent of its role in regulating primary T cell expansion, we needed a system in which we could circumvent the defects associated with the absence of MyD88 during initial LCMV infection. To achieve this, we made use of recently developed mice

in which the MyD88 gene has been floxed(B. Hou et al. 2008). While these mice had the potential to allow us to conditionally delete MyD88 in memory T cells, we first wanted to verify that deletion of MyD88 in naïve T cells using *Myd88*$^{flox/flox}$ mice produced results consistent with our findings in Chapter 3 using germ-line *Myd88-/-* mice. We therefore crossed *Myd88*$^{flox/flox}$ mice with mice expressing Cre recombinase under the CD4 promoter(Lee et al. 2001). In the resulting mice, which we will subsequently term *Myd88ΔT* mice, MyD88 expression is selectively deleted in all αβ T cells on the basis of Cre expression starting at the CD4+CD8+ double-positive stage of thymocyte development (Fig 4.2A).

We found greatly reduced CD8 T cell expansion in *Myd88ΔT* mice in response to LCMV infection, resulting in dramatically reduced numbers of CD44high CD8 T cells in the spleens of infected *Myd88ΔT* mice in comparison to *Myd88*$^{flox/flox}$ littermates that did not express Cre-recombinase ($p < 0.01$) (Fig. 4.2B). Strikingly, CD8 T cell numbers in *Myd88ΔT* were reduced almost to the extent of complete *Myd88-/-* mice, supporting our earlier results in Chapter 3 and suggesting that the contribution of MyD88 to T cell expansion during LCMV infection is largely T cell intrinsic. Consistent with our earlier findings, we found that the reduced number of CD44^{high} CD8 T cells correlated with a reduced frequency of LCMV-specific effector cells, as identified by tetramer staining, in all tissues ($p = 0.02$) (Fig. 4.2C). Assessment of antigen-specific IFNγ production following *ex vivo* restimulation with LCMV-derived peptides also supported the reduced frequency of LCMV-specific effectors amongst both the CD8 and CD4 T cell populations ($p < 0.01$) (Fig. 4.2D). Interestingly, ex vivo restimulation indicated a greater reduction

in the frequency of cells responding to the np396-404 peptide than to the gp33-41 peptide in *MyD88ΔT* mice, as we had observed in *Myd88-/-* mice. Overall, these findings confirm our earlier results, showing that MyD88 expression in T cells is critical for T cell expansion in response to LCMV infection, and establish that inducible deletion of MyD88 using $Myd88^{flox/flox}$ mice represents an appropriate model for examining the role of MyD88 during LCMV infection.

4.3 A system to conditionally delete MyD88

To generate a system in which we could circumvent the defects associated with the absence of MyD88 during initial LCMV infection we crossed $Myd88^{flox/flox}$ mice with transgenic mice expressing CreERT2 under transcriptional control of the ubiquitin promoter. To identify cells that underwent Cre-mediated deletion in these mice we further crossed them with R26RYFP Cre reporter mice in which the activity of Cre recombinase induces expression of YFP. To improve the efficiency of Cre-mediated deletion of MyD88 we used experimental mice in which only a single allele of MyD88 needed to be deleted as the other was knocked out in the germline ($Myd88^{flox/-}$ $R26R^{YFP}$ CreT2 mice, hereafter referred to as cKO). As controls we used mice in which one allele of MyD88 was floxed and the other was WT ($Myd88^{flox/+}$ $R26R^{YFP}$ CreT2, hereafter referred to as cHet) (Fig. 4.3).

Having generated these mice we wanted to confirm that tamoxifen treatment allowed the conditional deletion of MyD88 and that deleted cells could be identified on the basis of YFP expression. We therefore examined the effect of tamoxifen treatment on

the expression of MyD88 in splenocytes from cKO and cHet littermates. We found that tamoxifen treatment induced high expression of YFP in 25-50% of splenocytes in both cKO and cHet mice and that the proportion of YFP-expressing cells in the CD4 and CD8 T cell compartments was comparable to the proportion in the overall splenocyte population (Fig 4.4A). In order to determine whether YFP served as an accurate reporter of MyD88 expression, we sorted the YFP+ and YFP- fractions from tamoxifen treated cKO and cHet mice or untreated controls and performed a western blot to assess MyD88 protein levels. We found that the majority of YFP+ splenocytes in cKO mice had deleted MyD88 whereas there was minimal deletion in the YFP- compartment (Fig 4.4B). In contrast, MyD88 expression was comparable in YFP+ and YFP- splenocytes from cHet mice, suggesting that loss of a single *Myd88* allele does not appreciably reduced MyD88 protein expression. While this showed that tamoxifen treatment effectively induced MyD88 deletion in bulk YFP+ cKO splenocytes we also wanted to confirm that deletion occurred specifically in the T cell compartment. We therefore sorted YFP+ and YFP- CD4 and CD8 T cells from cKO mice and examined MyD88 expression by western blot. Consistent with the overall splenocyte population, we found that MyD88 was largely deleted in the YFP+ CD4 and CD8 T cells but was only minimally deleted in YFP- population (Fig 4.4C).

To further confirm that that YFP+ cells in cKO mice reproduce the phenotype that we had observed in germline *Myd88-/-* T cells and *Myd88ΔT* mice, we infected tamoxifen-treated cKO and cHet mice with LCMV. As expected, we observed significantly reduced expansion of the YFP+ CD8 T cells in cKO relative to cHet mice following LCMV infection ($p = 0.02$) (Fig 4.5A). In contrast, we did not observe a

significant difference in the expansion of the YFP- CD8 T cells in cKO relative to cHet mice ($p = 0.27$) (Fig 4.5B), supporting the limited deletion of MyD88 that we had observed in YFP- cKO T cells (Fig 4.4C). In further support of our finding that YFP- and YFP+ cells in cHet mice express comparable levels of MyD88 (Fig 3.3B) there was no significant difference between the expansion of YFP+ and YFP- CD8 T cells in cHet mice ($p = 0.39$), suggesting that heterozygous expression of MyD88 is sufficient to regulate T cell expansion in response to LCMV infection. Consistent with our earlier findings, the reduced CD8 T cell expansion in cKO mice correlated with a significantly reduced percentage of LCMV-specific effector cells in the YFP+ ($p < 0.02$) (Fig 4.5C), but not the YFP- CD8 T cell population ($p > 0.74$) (Fig 4.5D), as determined by antigen-specific IFNγ production. Furthermore, this reduced percentage of YFP+ IFNγ-producing cells in cKO mice reflected a significantly reduced frequency of antigen-specific cells as determined by tetramer staining ($p = 0.02$) (Fig. 4.5E).

Given that tamoxifen treatment of cKO presumably results in deletion of MyD88 in multiple immune compartments, the fact that we observe significant differences in the expansion of YFP- and YFP+ T cell populations in cKO mice is consistent with our earlier findings supporting a T cell-intrinsic role for MyD88. In addition, the finding that there is no significant difference between the expansion of YFP- T cell population in cKO and cHet mice supports our earlier mixed bone marrow chimera experiments (Fig. 3.5), which indicated that expression of MyD88 by half of the immune system is sufficient to support T cell responses against LCMV. Overall, these results demonstrate that tamoxifen treatment allows conditional deletion of MyD88 in cKO but not in cHet

mice and that YFP expression serves as an accurate reporter for MyD88 expression in cKO cells.

4.4 MyD88 is not required for T cell contraction and memory differentiation following LCMV infection

Having shown that the YFP+ T cells in cKO and cHet and effectively replicate the phenotype of germline *Myd88-/-* and *Myd88+/-* T cells following LCMV infection, we used matched cKO and cHet control mice to examine whether MyD88 plays a role in the contraction and differentiation of memory T cells independently of its role in initial expansion (Fig 4.6A). Following infection with LCMV-Arm we found no significant differences in the frequency of LCMV-specific CD8 T cells in the blood of cKO and cHet mice 8 days following infection ($p = 0.66$) (Fig 4.6B), confirming that *Myd88* is haplo-sufficient for regulating initial T cell expansion We then treated mice with tamoxifen on day 10 post infection to delete MyD88 after the peak of the CD8 T cell response. Serial bleeds showed that tamoxifen treatment induced a comparable frequency of YFP+ cells in the blood both cKO and cHet mice and that these cells were maintained stably following infection (Fig 4.6C). This indicates that rejection of deleted cells on the basis of their acquired expression of YFP is not a concern in this system.

We next examined the contraction and maintenance of LCMV-specific CD8 T cells following deletion of MyD88 by measuring their frequency in peripheral blood. Since we had found that MyD88 was only deleted effectively in the YFP+ population of cKO mice (Fig 4.4 and 4.5) we limited our analysis to YFP+ T cells. We found that the

populations of both gp33-specific (Fig 4.6D) and np396-specific (Fig. 4.6E) YFP+ CD8 T cells contracted with similar kinetics and were maintained at a comparable frequency in the blood of cKO and cHet mice for up to 60 days post infection ($p > 0.4$ for all time points).

One of the attributes that distinguishes memory T cells from naïve T cells is their migration through peripheral tissues and bone marrow((Mackay et al. 1990)(Slifka et al. 1997). Since *in vitro* studies have suggested that TLR stimulation may influence the expression of chemokines and integrins that regulate trafficking, we examined whether the loss of MyD88 influenced the trafficking of memory T cells. We found a comparable frequency of gp33-specific cells within the YFP+ lymphocyte population in the blood, spleen, lymph nodes, liver and bone marrow of cKO and cHet mice 60 days after LCMV infection ($p > 0.72$) (Fig 4.7A). This showed that the comparable frequency of LCMV-specific memory cells detected in the blood of cKO and cHet mice reflected a comparable representation in other compartments, suggesting that MyD88 is not required for normal homing and circulation of memory cells.

As discussed earlier, another attribute that distinguishes memory cells from naïve T cells is that they do not require TCR-derived signals for their maintenance and instead depend on the cytokines IL-7 and IL-15 for their homeostasis. It has recently been shown that stimulation through MyD88-dependent TLR pathways can promote memory T cell proliferation in response to IL-7(Cottalorda et al. 2009), raising the potential of decreased IL-7 responsiveness in MyD88-deficient memory T cells. However, consistent with their stable maintenance, we found that YFP+ LCMV-specific memory CD8 T cells in cKO

mice expressed comparable levels of CD127, CD122 and CD25 to those in cHet mice, suggesting normal recognition of these homeostatic cytokines (Fig 4.7B). In addition, we also found that YFP+ LCMV-specific memory cells in both cKO and cHet mice expressed a comparable distribution of CD62L, indicating that MyD88 is not required for differentiation into effector memory or central memory subsets (Fig 4.7B). We also noted that both cKO and cHet memory cells had downregulated PD-1 expression to the levels of naïve cells (Fig 4.7 B), further supporting a memory cell phenotype rather than a functionally exhausted phenotype. Overall, these findings indicate that, independent of its role in regulating primary expansion, MyD88 is dispensable for the differentiation, maintenance and migration of memory CD8 T cells following LCMV infection.

4.5 MyD88 is not required for memory T cell effector functions

As described earlier, another attribute of memory T cells is their ability to rapidly acquire effector functions in response to stimulation. It has recently been shown that the maintenance of memory T cells in a state of rapid responsiveness is dependent on the provision of adequate costimulatory signals through members of the tumor necrosis factor superfamily(Allam et al. 2009). Given that MyD88-dependent signals can serve as a T cell costimulatory pathway and TLR ligands can directly promote IFNγ secretion from memory T cells(Cottalorda et al. 2009), we examined whether the loss of MyD88 influenced the ability of cKO memory cells to rapidly produce effector cytokines.

60 days after LCMV infection, we found that a comparable frequency of YFP+ CD8 T cells in the splenocytes cKO and cHet mice produced IFNγ following a brief

restimulation with LCMV-derived peptides ($p > 0.4$) (Fig 4.8A). Effector cytokine production was not limited to IFNγ since a comparable frequency of YFP+ CD8 T cells in cKO and cHet mice also produced TNFα and IL-2 following restimulation (Fig. 4.8A). Furthermore, there was no significant difference in the overall distribution of memory cells that produced various combinations of these cytokines ($p > 0.2$) (Fig. 4.8B), suggesting that MyD88 is not required for the development of multifunctional memory cells We also found a comparable frequency of IFNγ and IL-2 producing cells in the YFP+ CD4 T cell compartment of cKO and cHet mice ($p > 0.3$) (Fig. 4.8A). These results indicate that MyD88-dependent signals are not required for the rapid production of effector cytokines by LCMV-specific memory T cells.

4.6 MyD88 is not required for memory T cell responses to secondary infection

Our results thus far indicate that T cell expression of MyD88 is required during initial expansion following LCMV infection, but is dispensable for the subsequent differentiation of functional memory cells. This raises the possibility that the MyD88-dependent survival signals in effector T cells are triggered by ligands that are only present during active LCMV infection, and that the lack of a role for MyD88 in memory cells merely reflects that the relevant ligands are no longer present since virus has been cleared. Alternatively, it is possible that, once naive cells have differentiated into LCMV-specific memory cells, signals through alternate pathways can compensate for the absence of MyD88-dependent signals. To distinguish between these possibilities, we next

examined whether MyD88 plays a role in controlling the survival and expansion of memory cells following secondary infection with LCMV.

Using cKO and cHet mice that had been infected with LCMV-Arm 60 days earlier and treated with tamoxifen following the primary response, we induced secondary infection with a high dose of LCMV-CL13. Reinfection induced a rapid expansion of CD8 T cells in both cKO and cHet mice. Notably, we observed a significantly lower frequency of YFP+CD44+ CD8 T cells in the blood of cKO than cHet mice 3 days following reinfection, however there was no longer a significant difference by day 6 (Fig 4.9A). Surprisingly, we detected minimal expansion of the gp33-specific population in the blood of either cKO or cHet mice, but did find robust expansion of the np396-specific population (Fig. 4.9B), which has been reported to dominate secondary responses(Tebo et al. 2005). Consistent with the pattern in overall CD44+ cells, there was a significantly lower frequency of YFP+ np396-specific cells in the blood of cKO mice 3 days post reinfection, but both cKO and cHet mice contained a comparable frequency by 6 days post reinfection. This was mirrored by a comparable frequency of LCMV-specific CD8 T cells in the spleens of cKO and cHet mice 6 days post infection ($p = 0.30$) (Fig. 4.9C).

Since we had observed increased apoptosis in *Myd88-/-* effector cells during primary expansion in response to LCMV, we examined the survival of LCMV-specific T cells following secondary expansion. Consistent with their comparable expansion, we found no notable differences in the apoptosis of YFP+ np396-specific cells in cKO and cHet mice either directly *ex vivo*, or following *in vitro* culture (Fig. 4.9D).

While these data suggested effective expansion and survival of MyD88-deficient memory T cells, it was possible that the secondary T cell response observed in cKO mice was due to the outgrowth of a population of YFP+ CD8 T cells that had failed to delete MyD88. We therefore sorted the YFP+ and YFP- fractions from the CD44+CD8 T cell populations in cKO and cHet mice and examined MyD88 expression. We found that the majority of YFP+ cells in cKO mice still lacked MyD88 expression, indicating that the memory T cell response in cKO mice did not merely reflect preferential expansion of MyD88-sufficient cells (Fig 4.9E). It should also be noted that some of the MyD88 expression in the YFP+ cKO sample may represent contaminating YFP- cells since the sort purity of this sample was only 80%.

We also considered whether MyD88 may potentially be involved in the differentiation of memory cells into functional secondary effectors. *Ex vivo* restimulation with LCMV-derived peptides indicated a comparable frequency of IFNγ producing cells within the YFP+ CD8 T cell compartments of cKO and cHet mice (4.10A), as well as a similar frequency and distribution of cells that produced combinations of IFNγ, TNFα and IL-2 ($p > 0.1$) (4.10B). Interestingly, in contrast to our results using gp33 tetramers, we found that secondary LCMV infection induced a considerable expansion in the population of cells that produced IFNγ in response to restimulation gp33-41 in both cKO and cHet mice. This could indicate preferential expansion of cells recognizing the epitope in the context of H2-K^b during the secondary immune response to LCMV. Overall, these data indicate that, while MyD88-expression may contribute slightly to the kinetics of early memory T cell expansion following secondary LCMV infection, the MyD88-

dependent signals induced by LCMV infection are not critical for memory T cells to achieve maximal expansion or differentiation at the peak of the secondary response.

Summary

It is well established that memory T cells are functionally and phenotypically distinct from naïve and effector T cells. For example, memory T cells rely on the cytokines IL-7 and IL-15, rather than TCR-induced signals for their homeostasis and concordantly express higher levels of the IL-7R and IL-15R than naïve T cells. Studies have also found that memory T cells constitutively expresses higher levels of TLRs and are more responsive to TLR ligands than naïve T cells, thus raising the possibility that MyD88-dependent pathways may play a more important role in regulating memory T cell responses. However, our results in this chapter indicate that, following the primary response to acute LCMV infection, LCMV-specific T cells do not require MyD88-dependent signals to support their differentiation into memory cells or their subsequent maintenance. Furthermore, in contrast to the critical requirement for MyD88 in supporting naïve T cell expansion in response to primary LCMV infection, memory T cells expand and differentiate effectively following secondary encounter with LCMV in the absence of MyD88. Thus, differential requirements for MyD88-dependent signaling appear to represent another attribute that functionally distinguishes memory T cells from naïve T cells.

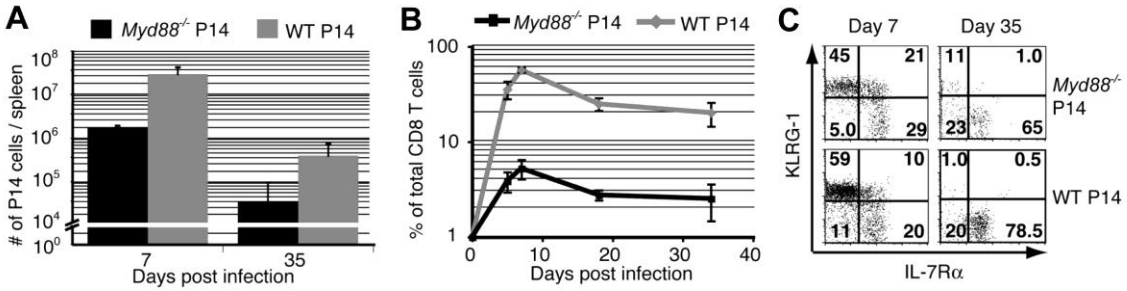


Figure 4.1. *Myd88-/-* P14 cells generate fewer memory cells following LCMV infection. *Myd88*-/- or WT P14 cells were transferred into individual WT recipients, which were infected 18hrs later. (A) On day 7 and day 35 post infection the numbers of WT and *Myd88*-/- P14 cells per spleen were enumerated. Data represent mean + S.D of 9 mice and $p < 0.01$ for both time points. (B) LCMV-infected recipients were longitudinally bled and the percentage of WT and *Myd88*-/- P14 cells was determined relative to total CD8 T cells. Data represent mean +/- SD of at least 9 mice and $p < 0.01$ for all time points. (E) Expression of KLRG-1 and IL-7Rα by WT and *Myd88*-/- P14 cells in day 7 and day 35 infected spleens. Data are representative of 5 mice and differences were not statistically significant.

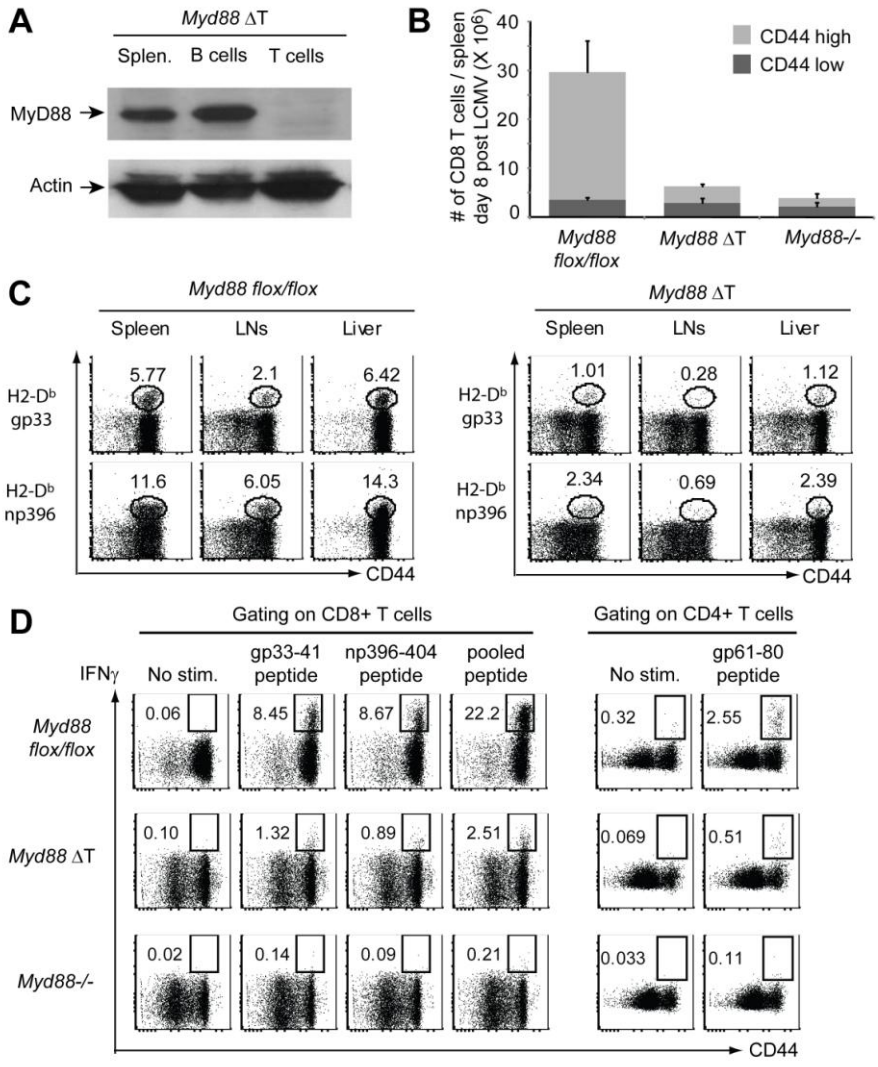


Figure 4.2. *Myd88ΔT* mice mount greatly reduced T cell responses to LCMV infection. (A) T and B cells were FACS purified from the spleen of a *Myd88ΔT* mouse and MyD88 expression was examined by Western blot. (B-D) *Myd88flox/flox*, *Myd88ΔT* and *Myd88-/-* mice were examined 8 days after LCMV infection. (B) The number of splenic CD8 T cells was determined. Data represent mean + SD of 4 mice per group. (C) The frequency of tetramer-specific cells amongst gated CD8 T cells from the indicated tissues was measured. (D) Splenocytes were restimulated with the indicated peptides and antigen-specific IFNγ production was assessed.

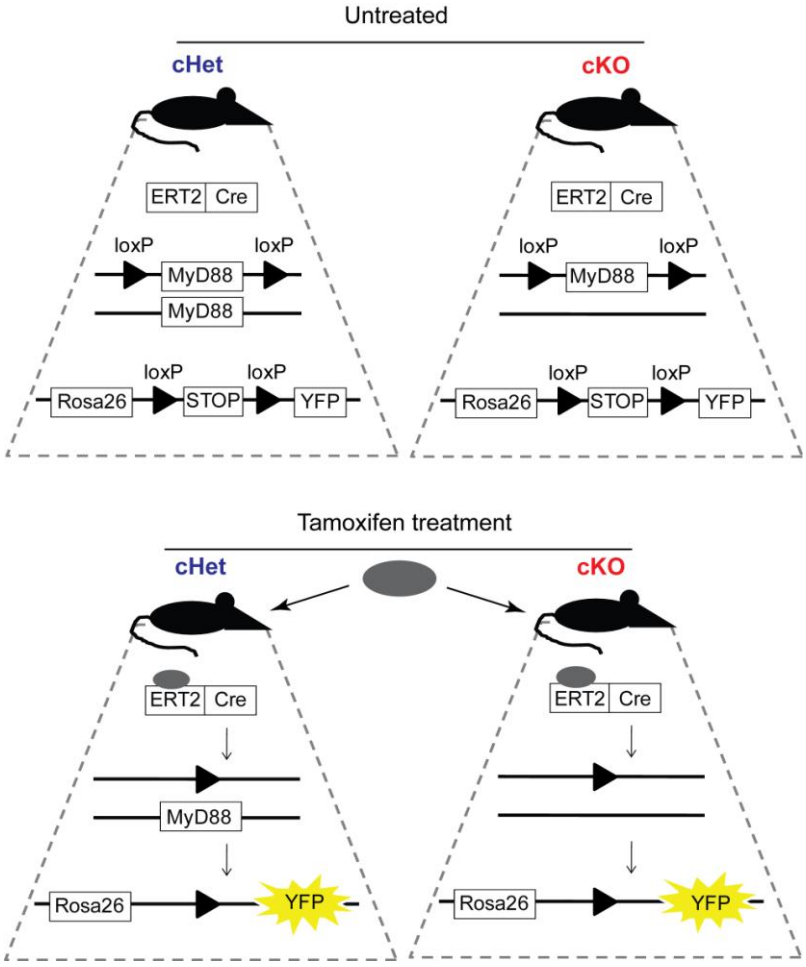


Figure 4.3. Schematic of the predicted outcome of tamoxifen treatment in cKO and cHet mice

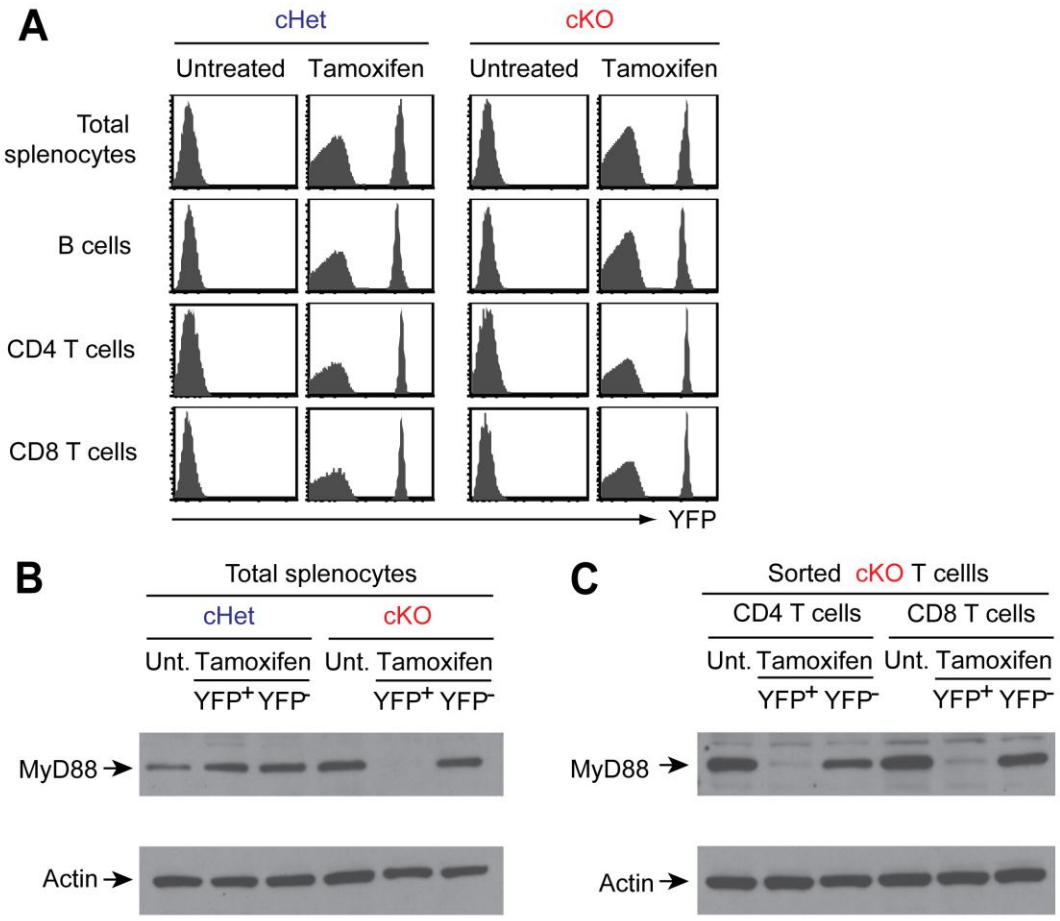


Figure 4.4. Tamoxifen treatment allows deletion of MyD88 correlated with YFP expression in cKO mice. (A) cHet mice cKO mice were treated with tamoxifen or left untreated and YFP expression in the indicated splenocyte populations was determined 5 days later. (B) YFP+ and YFP- splenocytes were FACS-purified from treated or untreated cHet and cKO mice. MyD88 expression was determined by Western blot. (C) YFP+ and YFP- CD4 and CD8 T cells were FACS-purified from treated or untreated cKO mice and MyD88 expression was determined by Western blot.

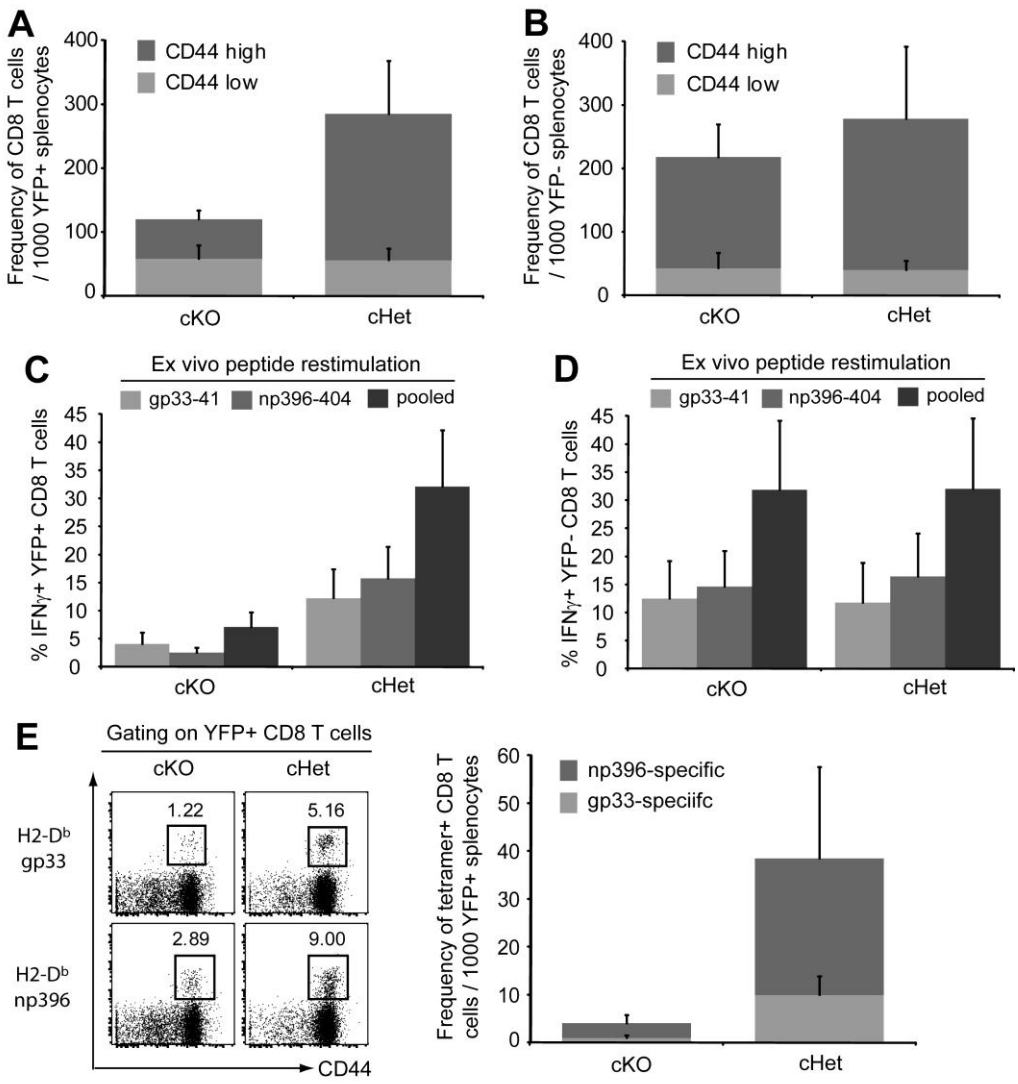


Figure 4.5. YFP+ T cells in cKO mice exhibit reduced expansion in response to LCMV infection. cKO and cHet mice were treated with tamoxifen and infected with LCMV-Arm 5 days later. Splenocytes were examined 8 days after infection. (A-B) The frequency of CD8 T cells in the YFP+ (A) and YFP- (B) splenocyte populations was determined. (C-D) Splenocytes were restimulated with the indicated peptides and the frequency of IFNγ-producing YFP+ (C) and YFP- (D) CD8 T cells was determined by intracellular staining. (E) The frequency of LCMV-specific YFP+ CD8 T cells was determined using tetramers. All graphs represent mean + SD of at least 4 mice.

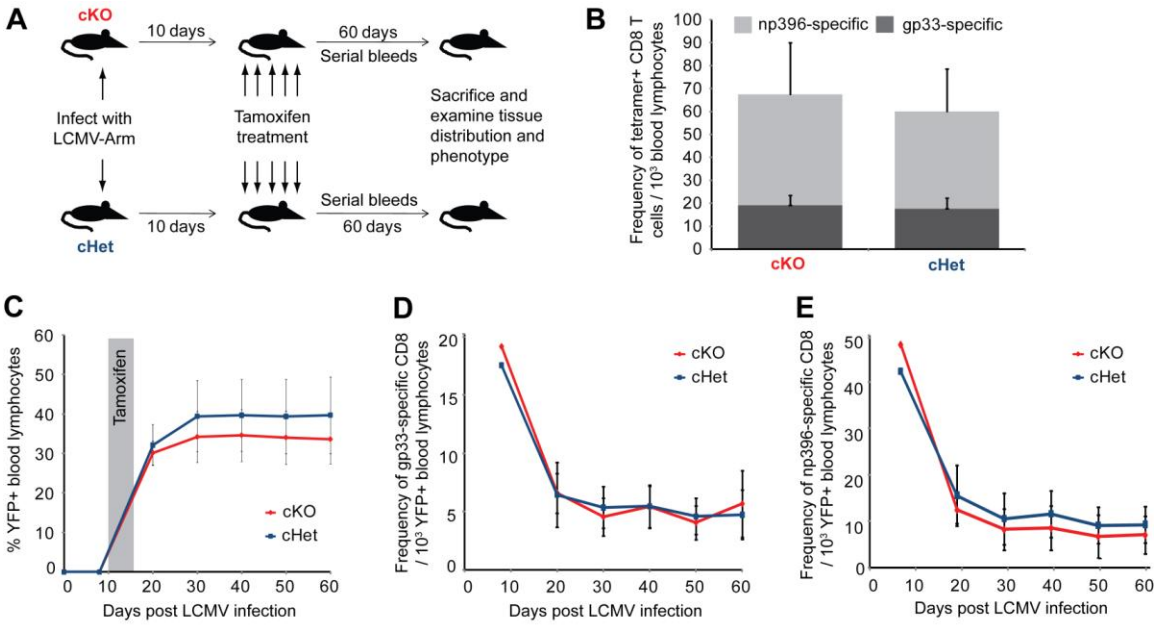


Figure 4.6. MyD88 is not required for the development and maintenance of memory T cells following LCMV infection. (A) Experimental design. (B) The frequency of tetramer-specific CD8 T cells in the blood of cKO and cHet mice was determined 8 days after LCMV infection. Data represent mean + SD of at least 6 mice. (C) The frequency of YFP+ cells was measured in blood lymphocytes of cKO and cHet mice by serial bleeds. Data represent mean +/- SD of at least 6 mice. (D-E) The frequency of gp33-specific (D) and np396-specific (E) cells within YFP+ blood lymphocytes of cKO and cHet mice was determined by tetramer staining. Data represent mean +/- SD of at least 6 mice.

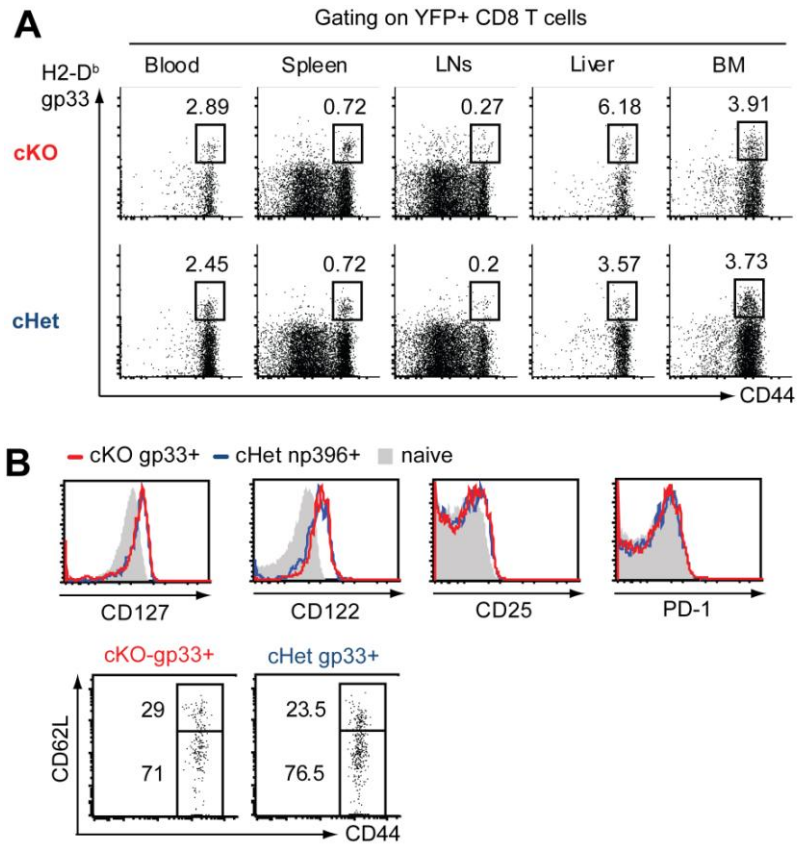


Figure 4.7. MyD88 is not required for normal T cell tissue homing or the acquisition of a memory phenotype following LCMV infection. cKO and cHet mice were infected with LCMV-Arm and treated as in Figure 4.6 (A) The frequency of gp33-specific cells within the YFP+ CD8 T cells from the indicated tissues of cKO and cHet mice was assessed by tetramer staining 60 days after infection. (B) The expression of the indicated phenotypic markers was determined on gated gp33-specific YFP+ cells from the spleens of cKO and cHet mice 60 days after infection.

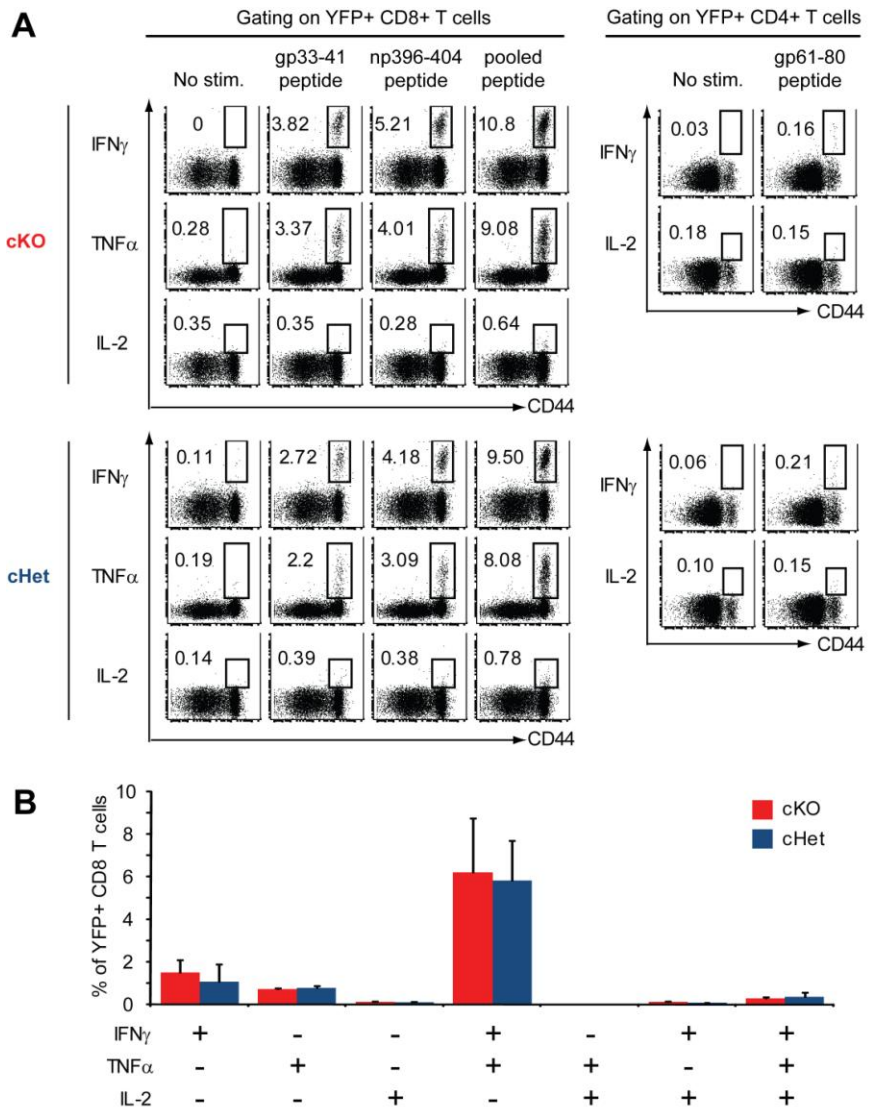


Figure 4.8. MyD88 is not required for the rapid production of effector cytokines by memory T cells. (A) Splenocytes from cKO and cHet mice 60 days following LCMV infection were restimulated with the indicated LCMV-derived peptides and IFNγ, TNFα and IL-2 production by YFP+ CD8 T cells was assessed by intracellular staining. (B) The frequency of YFP+ CD8 T cells producing the indicated combinations of cytokines in response to stimulation with pooled LCMV peptide was determined. Data represent mean + SD of 3 mice.

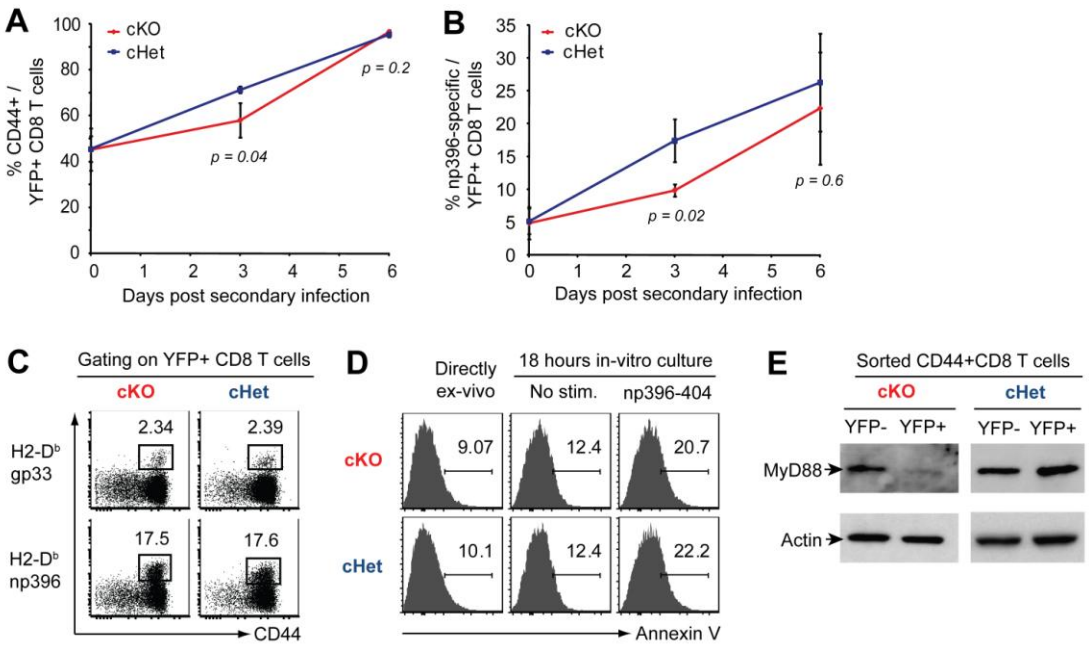


Figure 4.9. MyD88 is not critical for memory T cell expansion in response to secondary LCMV infection. cKO and cHet mice were infected with LCMV-Arm and treated as in Figure 4.6, and were reinfected with LCMV-CL13 60 days after primary infection. (A-B) The frequency of CD44+ (A) and np396-specific (B) YFP+ CD8 T cells in the blood at the indicated time points was determined by tetramer staining. Data show mean +/- SD of 3 mice. (C) The frequency of tetramer specific YFP+ CD8 T cells in the spleens of cKO and cHet mice was assessed 6 days post reinfection. (D) YFP+ and YFP- CD44+CD8 T cells were sorted from the spleens of reinfected cKO and cHet mice and MyD88 expression was measured by Western blot. (D) The viability of gated np396-specific YFP+ CD8 T cells from cKO and cHet mice was assessed by Annexin V staining direct either *ex vivo* or after the indicated 20 hours of *in vitro* culture as indicated. Data are representative of 3 mice per group.

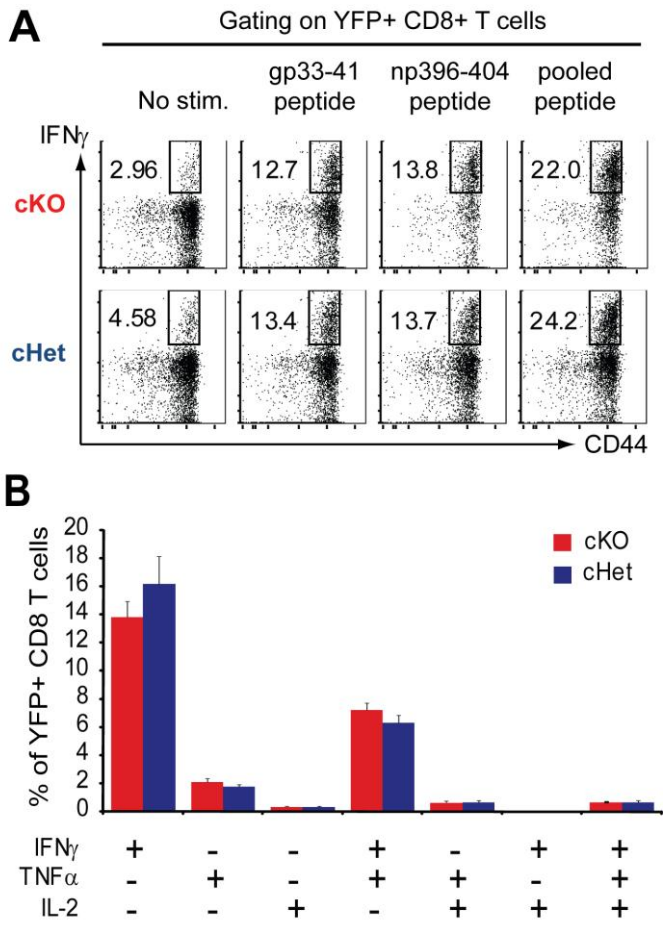


Figure 4.10. MyD88 is not required for the differentiation of memory T cells into secondary effectors. LCMV immune cKO and cHet mice were reinfected with LCMV Cl-13 as in Figure 4.9. (A) 6 days after reinfection, splenocytes were restimulated with the indicated LCMV-derived peptides and IFNγ, TNFα and IL-2 production by YFP+ CD8 T cells was assessed by intracellular staining. (B) The frequency of YFP+ CD8 T cells producing the indicated combinations of cytokines in response to stimulation with pooled LCMV peptide was determined. Data represent mean +SD of 3 mice per group.

CHAPTER 5: Discussion and Speculation

Defining the role of MyD88 in T cells: contrasting the findings of *in vitro* and *in vivo* studies

A growing number of studies have recently shown that TLR ligands can act directly on mouse and human T cells to promote their proliferation, survival and differentiation *in vitro*, challenging the prevailing view that suggested that the importance of TLRs was restricted to cells of the innate immune system. These findings raised the potential that the defective T cell responses against numerous pathogens that had been observed in MyD88 mice may reflect a direct role for MyD88-dependent signaling in T cells. However, the relative contributions of T cell extrinsic and intrinsic MyD88-dependent signals and, thus, the overall physiological relevance of MyD88-expression in T cells, during a natural *in vivo* immune response remained unclear.

The findings presented in this dissertation being to address these issues by using LCMV infection as model viral pathogen to examine the role of MyD88 during *in vivo* T cell responses. We confirm that MyD88 plays an important T cell-extrinsic role in regulating T cell responses, as evidenced by reduced expansion and a shift in immunodominance during the response of WT CD8 T cells to LCMV infection in a *Myd88-/-* host (Chapter 3.3). This supports the reduced production of type I IFNs and other pro-inflammatory cytokines by *Myd88-/-* APCs(Zhou et al. 2005)(Jung et al. 2008), which, in turn, have been shown to be important in regulating T cell expansion(Kolumam et al. 2005)(Aichele et al. 2006). However, our findings importantly show that MyD88 also plays a critical, T cell-intrinsic role in supporting the survival and accumulation of antigen specific CD8 T cells during the response to acute LCMV infection.

During the course of this dissertation work, a number of other studies have expanded upon earlier *in vitro* work to examine the potential role of TLR and MyD88-dependent signaling in T cells during *in vivo* immune responses. As discussed earlier, *Myd88*-/- mice display significantly greater mortality following infection by the protozoan pathogen, *Toxoplasma gondii*(Scanga et al. 2002). This increased susceptibility is associated with reduced Th1 effector responses, which have been attributed to defective innate immune activation and reduced levels of IL-12 production in these mice. In a recent study, our lab found that mixed bone marrow chimeras in which MyD88-deficiency was largely restricted to the T cell compartment generated reduced numbers of IFNγ-producing effector T cells following *T. gondii* infection and were highly susceptible to toxoplasmic encephalitis, succumbing to infection with similar kinetics to full *Myd88*-/- mice(LaRosa et al. 2008). These findings indicate that MyD88 expression in T cells is required for prolonged resistance to *T. gondii* in the setting of an intact innate immune response but did not establish a mechanism by which MyD88 contributes to T cell responses in this model.

MyD88 expression in T cells was also found to play a role in CD4 T cell-mediated colitis. When CD4+CD45RB^{high} T cells are transferred into RAG1/2-deficient animals, the recipients rapidly develop wasting disease associated with clinical signs of colitis(Powrie et al. 1993)(Morrissey et al. 1993). This experimental colitis depends on the presence of commensal intestinal microflora and TLRs from these microorganisms are thought to activate innate immune cells in the gut and thereby indirectly support colitogenic T cell responses(Cong et al. 1998)(Sartor 2001). However, it has recently been

reported that purified *MyD88*-/- CD4+CD45RB^{high} T cells transferred into RAG-deficient recipients do not induce as severe colitis as WT T cells(Tomita et al. 2008)(Fukata et al. 2008). Using competitive transfer systems, *Myd88*-/- colitogenic CD4 T cells were shown expand less effectively than WT cells and this correlated with reduced production of effector cytokines. In particular, *MyD88*-/- CD4 T cells isolated from the lamina propria produced significantly less IL-17. Furthermore, it was shown that *Myd88*-/- CD4 T cells did not effectively differentiate into IL-17-producing cells *in vitro*, suggesting that MyD88 may play a role in Th17 polarization(Fukata et al. 2008). Relevant to this, *Myd88*-/- mice are highly resistant to the induction of experimental autoimmune encephalitis (EAE), which is an IL-17 mediated autoimmune disease(Marta et al. 2008). While reduced numbers of *Myd88*-/- Th17 cells in this model have been attributed to decreased production of IL-6 and IL-23 by *Myd88*-/- dendritic cells, it is interesting to consider whether they also reflect a T cell-intrinsic role for MyD88 in supporting Th17 responses.

These studies using *T. gondii* and colitis models demonstrated that MyD88-dependent signals in T cells are a physiologically relevant component of T cell responses *in vivo*. However, since they were only able to examine overall polyclonal T cell responses, these studies were limited in their ability to directly compare antigen-specific populations of WT and *MyD88*-/- effector T cells. Thus, while reduced levels of CFSE dilution and BrdU uptake were identified in *MyD88*-/- colitogenic CD4 T cell populations, it remained unclear whether this represented reduced proliferation of antigen-specific T cell clones or increased cell death resulting in a smaller proportion of dividing colitogenic cells. Similarly, it was unclear whether impaired cytokine production by *MyD88*-/- T cell populations in these models represented impaired effector

differentiation, reduced cytokine secretion by antigen-specific responders, or a relatively smaller proportion of cytokine-producing effector cells.

The findings presented in this dissertation support and expand upon these studies to further define the role of MyD88 in T cells during viral infection. The use of LCMV as an infection model allowed us to employ epitope-specific cytokine production, MHC Class I tetramers and P14 TCR-transgenic mice to directly compare the responses of WT and MyD88-deficient antigen-specific T cells. Our results in Chapter 3, using adoptive cells transfers and bone marrow chimeras, clearly demonstrate that MyD88 plays a T cell-intrinsic role in supporting T cell expansion, but not effector differentiation, in response to LCMV infection. While this reduced expansion is associated with decreased BrdU incorporation in the overall *Myd88-/-* CD8 T cell population, examination of LCMV-specific effector cells allow us to attribute the reduced expansion to impaired survival rather than impaired proliferation. Interestingly, examination of the polyclonal T cell response indicated the absence of MyD88 resulted in a more severe reduction in np396-specific than gp33-specific T cells. This may relate to the fact that T cells responding to the np396 epitope express higher levels of PD-1 and have been found to be in a more apoptotic state than those responding to gp33 epitope(X. Z. Wang et al. 2004), and may thus be more dependent on MyD88-dependent signals to rescue them from apoptosis.

Thus, while *in vitro* studies have suggested that MyD88-depndent TLR signals can potentially regulate CD8 T cell proliferation, survival and effector differentiation(Cottalorda et al. 2006), our results in Chapter 3 indicate that naïve CD8 T

cells can be activated and induced to proliferate and differentiate into functional and phenotypic effector cells during the early stages of the response to LCMV in the absence of MyD88. Instead the importance of MyD88 is limited to a critical role in supporting their subsequent survival and sustained accumulation, thereby controlling the ultimate size of the T cell effector population at the peak of the response (Fig. 5.1). Thus, MyD88-dependent signals appear to act in an analogous fashion to inflammatory cytokines, which serve as a third signal, in addition to TCR and costimulatory receptor engagement, in promoting optimal T cell expansion(Curtsinger et al. 2005). Our results are supported by recent studies that also indicate a similar role MyD88 in antigen-specific T cells during responses to LCMV and vaccinia virus infection(Bartholdy et al. 2009)(Quigley et al. 2009)(Zhao et al. 2009). This suggests that the defects associated with *Myd88-/-* T cells in the *T. gondii* and colitis models may also primarily be explained by reduced effector cell survival rather than impairments in proliferation or effector cell differentiation, though it is difficult to extrapolate findings between these experimental systems since the precise nature of the MyD88-mediated signals may vary.

In vitro studies have also suggested that MyD88-dependent TLR signals may contribute to the differentiation, homeostasis and reactivation of memory T cells(Mercier et al. 2009)(Cottalorda et al. 2009). We find that MyD88 expression in T cells is important in regulating the size of the resulting memory population following LCMV infection and a similar finding has been reported following vaccinia virus infection(Quigley et al. 2009). However, this likely reflects an indirect result of the importance of MyD88 in supporting initial T cell expansion, since initial antigen encounter and the number of effector cells generated during the primary response are known to regulate the differentiation and

ultimate size of the memory population(Hou et al. 1994)(Shaulov & Murali-Krishna 2008). Indeed, our results in Chapter 4 indicate that, independent of its role in the initial phase of T cell expansion, MyD88-expression is not required for the subsequent differentiation and maintenance of functional memory T cells. Furthermore, memory T cells appear to be less dependent on MyD88-dependent signals than naïve T cells for their expansion in response to secondary LCMV infection. Similarly, memory T cells induced in *Myd88-/-* mice by vaccination with gp33-expressing adenovirus were also shown to expand effectively in response to LCMV infection(Bartholdy et al. 2009). Thus, while *in vitro* studies suggest a role for MyD88-dependent signals in regulating memory T cell homeostasis and responses, it appears this these signals are not critical during *in vivo* responses to LCMV infection.

A consideration of the potential mechanisms for MyD88-mediated T cell survival during infection

Perhaps the most notable difference between our results and those suggested by *in vitro* studies is that the role of MyD88 during LCMV infection does not appear to be attributable to a specific TLR pathway. While TLR2 and TLR9 are the only MyD88-dependent TLR pathways that have been found to stimulate mouse CD8 T cells *in vitro*, neither *Tlr2-/-* nor *Tlr9-/-* mice recapitulated the impaired T cell expansion observed in *Myd88-/-* mice following LCMV infection. Indeed, *Tlr2-/-* mice exhibited significantly increased numbers of LCMV-specific cells in their spleens at the peak of infection. This surprising finding could potentially be related to the reduced frequency of T_{regs} reported

in these mice(Netea et al. 2004) or to reduced recruitment of T cells into other tissues due to impaired chemokine production(Zhou et al. 2008). It has been shown that mice deficient in TLR3, TLR4, and TLR8 mount normal CD8 T cell responses to LCMV, and while LCMV-specific CTL numbers are slightly reduced in mice lacking both TLR7 and TLR9, they do not recapitulate the phenotype of *Myd88-/-* mice (Jung et al. 2008).

MyD88 is also involved in signaling downstream of the IL-1R and IL-18R, and these cytokines have been shown to play a role in promoting CD8 T cell survival(Wen Li et al. 2007). However, we found no reduction in LCMV-specific CD8 T cells in the spleens of *IL1r1-/-* mice and somewhat reduced numbers in *IL18r1-/-* mice, which still greatly exceeded those found in *Myd88-/-* mice, suggesting that these pathways also do not account for the importance of MyD88 during LCMV infection. IL-33, which signals through ST2, has also recently been described as another IL-1R family cytokine. IL-33 signaling has been implicated in the polarization of T_H2 CD4 T cells(Schmitz et al. 2005), but has not been described to play a role in CD8 T cells. While this suggests that IL-33 is unlikely to account for the impaired responses of *Myd88-/-* CD8 T cells, its potential role cannot yet be ruled out.

Thus, while MyD88 clearly plays an important role in T cells during responses to LCMV infection, the specific upstream initiators of MyD88-mediated signaling remain unclear. Similarly, the T cell impairments associated with MyD88-deficiency during *T. gondii* infection cannot be attributed to a role for individual TLRs or IL-1 and IL18(Scanga et al. 2002)(Debierre-Grockiego et al. 2007)(Hitziger et al. 2005)(Yarovinsky et al. 2005)(LaRosa et al. 2008). In addition, while the role of MyD88 in vaccinia virus specific T

cells has been attributed to TLR2 signaling in one study(Quigley et al. 2009), another was unable to find such an association(Zhao et al. 2009). This suggests that an inability to define a role for specific TLR/cytokine pathways may be a consistent feature when considering the importance of MyD88 in T cells during *in vivo* immune responses. One possible explanation for this is that there is considerable redundancy and cooperation between different MyD88-dependent pathways *in vivo*, as has been noted in certain infections (Sørensen et al. 2008). Another, non-mutually exclusive, possibility is that the importance of MyD88 in CD8 T cells during infection is linked to novel upstream receptors or a function outside of its traditional role in TLR signaling.

In support of this latter possibility, while TLR-induced survival of T cells is associated with clear upregulation of Bcl-xL expression *in vitro*(Cottalorda et al. 2006), we did not detect significant differences in the levels of this pro-survival factor between WT and *Myd88-/-* CD8 T cells following LCMV infection. Instead the results of a full genome microarray suggested that the reduced survival of *Myd88-/-* cells may be related to differences in the expression of genes related to inositol phosphate metabolism, calcium signaling and amino acid metabolism. Though the significance of these findings is somewhat unclear, it is interesting to note that a potential connection between MyD88-dependent signaling pathways and regulation of cellular metabolism is suggested by a recent study that found that TRAF6-deficient CD8 T cells exhibit alteration of genes regulating fatty acid metabolism following *Listeria monocytogenes* infection(Pearce et al. 2009).

In considering other potential roles for MyD88 outside of TLR signaling we noted that MyD88 has been found to associate with the Fas-related signaling molecule, FADD in fibroblasts and macrophages. As a result of homotypic death-domain interactions, expression of FADD can inhibit signaling through TLRs by sequestering MyD88 away from TLR signaling pathways(Zhande et al. 2007). This raised the reciprocal possibility that loss of MyD88 may allow excess FADD to be recruited to the Fas signaling pathway, thereby rendering *Myd88-/-* T cell hyper-responsive to Fas induced apoptosis. However, we did not find that *Myd88-/-* T cells had increased sensitivity of Fas or TNF induced AICD *in vitro*, suggesting that these pathways do not account for the reduced survival of *Myd88-/-* T cells following LCMV infection. However, an interaction of MyD88 with other death-domain containing proteins remains a possibility.

It is also interesting to note the striking similarity in the apparent roles of Type I IFN and MyD88-dependent signals in T cells during LCMV infection. Like *Myd88-/-* T cells, IFN-IR-/- T cells proliferate and differentiate but exhibit reduced survival, resulting in dramatically reduced expansion following LCMV infection(Aichele et al. 2006)(Kolumam et al. 2005). While this similarity in phenotype may be coincidental, it is tempting to speculate that it may reflect an intersection between the IFN-IR and MyD88 signaling pathways. As discussed earlier, MyD88 is known to play an important role in promoting the production of Type I IFNs through the induction of IRF-dependent signaling pathways(Taro Kawai et al. 2004). However, a potential role for MyD88 downstream of Type I IFN induced signaling pathways is less clear. TLR signaling has been shown to promote STAT1 phosphorylation in macrophages, raising the potential that MyD88 may be involved in STAT-dependent cytokine responses(Rhee et al. 2003).

However, given that TLR signaling has been shown induce the production of IFNα in APCs, this finding may reflect a secondary outcome of increased IFNα production. In support of this, we did not observe defects in STAT phosphorylation or general responses to Type I IFNs in *Myd88-/-* CD8 T cells, though this does not rule out a more subtle role for Type I IFNs in promoting MyD88-dependent signals or vice versa.

Notably, similarly to IFN-IR-/- CD8 T cells, IFNγR1-/- CD8 T cells have also been shown to exhibit reduced expansion in response to LCMV infection(Whitmire et al. 2005), though the mechanisms by which IFNγ directly promotes CD8 T cell abundance remain unclear. Interestingly, MyD88 has been shown to physically interact with the IFNγR1 and thereby stabilize IFNγ-induced mRNA transcripts in macrophages(Sun & Ding 2006). This raises the intriguing possibility that MyD88 may function similarly in CD8 T cells, suggesting that the reduced survival of *Myd88-/-* CD8 T cells following LCMV infection reflects impaired expression of IFNγ-induced survival genes. Furthermore, a similar association between MyD88 and the IFN-IR remains an unexplored possibility.

In considering the role of Type I IFNs in regulating T cell responses, it is notable that T cells appear to be particularly dependent on IFN-IR-mediated signals for their clonal expansion in the setting of LCMV infection. In contrast, T cell expansion in response to vaccinia, VSV and *Listeria monocytogenes* infection is only mildly impaired in the absence of IFN-IR signaling(Thompson et al. 2006). Correspondingly, while MyD88-dependent signals are critical for T cell survival in the setting of LCMV and vaccinia infection(Quigley et al. 2009)(Zhao et al. 2009), it is interesting to note that there is no significant reduction in the expansion of *Myd88-/-* T in response to VSV(Bartholdy et

al. 2009) and only moderately impaired expansion in response to adenovirus(Bartholdy et al. 2009) and Listeria infection (S. M. Kaech, unpublished observations). Furthermore, while we have found that MyD88-expression in T cell is required for resistance to *T. gondii*(LaRosa et al. 2008), it is notable that loss of MyD88 results in a far smaller reduction in effector CD8 T cell frequency during *T. gondii* infection than LCMV infection.

Thus, it appears that MyD88-dependent signals are not universally required for promoting T cell expansion and their importance is instead linked to certain infections. This could indicate that the upstream ligands that trigger the relevant MyD88-dependent pathways in T cells are only encoded by, or induced in the setting of, certain pathogens. Alternatively, and non-exclusively, it is possible that other characteristics of the infection dictate the relative importance of MyD88-dependent signals in contributing to overall T cell survival. Due to its non-cytopathic nature, LCMV provides a very large antigenic load, which is an important contribution to the dramatic T cell expansion induced by this virus (Thompson et al. 2006). In addition, studies using different strains of LCMV indicate that the *Myd88-/-* T cell response is more significantly impaired following infection high-dose with the invasive LCMV-Traub strain than the acute LCMV-Arm strain(Bartholdy et al. 2009). This could indicate that an environment of prolonged antigenic load is an important factor in determining the importance of MyD88-dependent survival signals. Consequently, the more severe impairment in the np396-specific responses may be related to higher antigen loads of this immunodominant epitope(van der Most et al. 2003). This could also suggest that the relatively minor role for MyD88-expression in memory T

cell expansion following secondary LCMV infection is related to the relatively rapid clearance of virus. To further explore this possibility we are interested in examining the role of MyD88-dependent signals in regulating T cell survival and maintenance in the setting of persistent antigen exposure induced during chronic LCMV infection(Wherry et al. 2003).

While this potential avenue of investigation would serve to further define the role of MyD88 in T cells during infection, additional insight would be gained by identifying the critical upstream and downstream mediators that are responsible for conferring the ability of MyD88 to promote T cell survival during LCMV infection. As discussed earlier, MyD88 is known to engage upstream receptors through homotypic TIR domain interactions and subsequently recruit IRAK4 through homotypic death domain interactions ultimately leading to NFκB activation(Wesche et al. 1997)(Martin & Wesche 2002). However, additional studies have found that MyD88 is also able to activate the Akt pathway through a direct interaction of phosphatidylinositol 3-kinase (PI-3-K) with a tyrosine motif in the MyD88 TIR domain(Li et al. 2003)(Rhee et al. 2006)(Gelman et al. 2006). At present it is unclear which, if any, of these pathways is responsible for the MyD88-induced survival effect during LCMV infection.

The fact that TLR stimulation can promote Akt phosphorylation *in vitro*, and that Akt signaling is required for T cell responses to vaccinia has led others to conclude that Akt activation represents the critical MyD88-dependent pathway during infection, though this has not been directly demonstrated(Quigley et al. 2009). Indeed, we have found that the Akt pathway primarily contributes to TLR-induced T cell proliferation rather than

survival(Gelman et al. 2006), which is inconsistent with our finding that *Myd88-/-* T cells have a specific defect in survival rather than proliferation. Furthermore, the fact that mice lacking IRAK4, the signaling kinase downstream of MyD88, also exhibit reduced T cell expansion in response to LCMV(Lye et al. 2008) suggests that this may represent the relevant pathway during LCMV infection. However, this hypothesis is complicated by the finding that T cells lacking TRAF6, the signaling molecule downstream of IRAK4, do not exhibit impaired expansion but rather impaired memory differentiation following Listeria infection(Pearce et al. 2009), contrasting with the phenotype of *Myd88-/-* T cells. In addition, since Bcl-xL expression is upregulated as a result of MyD88-induced NF-κB activation, the fact that we did not observe differences in the expression of Bcl-2 family members further questions the role of this pathway in regulating T cell survival during LCMV infection.

In considering how to clarify some of these issues, it is useful to note that studies have begun to identify the molecular motifs associated with MyD88 TIR and Death domain interactions(Ohnishi et al. 2009)(Chunsheng Li et al. 2005)(Loiarro et al. 2009)(Gelman et al. 2006). It is therefore potentially feasible to generate mutated forms of MyD88 that are unable to initiate specific signaling pathways. By transfecting *Myd88-/-* deficient cells with mutant forms of MyD88, the relative importance of various domains of MyD88 in promoting T cell survival during LCMV infection could be selectively assessed. This approach could help to clarify the critical binding partners of MyD88 and elucidate the relevant signaling pathways, thereby establishing a more comprehensive mechanism for the physiological role of MyD88 in T cells.

Conclusions

MyD88-dependent signaling through TLRs has long been recognized to play an important, indirect role in supporting T cell responses through myriad effects on innate immune cells, including up-regulating antigen presentation, co-stimulatory molecule expression and inflammatory cytokine production. In addition to these importance effects in cells of the innate immune system, it is now clear that MyD88-dependent TLR ligands can also directly on T cells. Several *in vitro* studies have shown that TLRs can potentially serve as co-stimulatory receptors on T cells, and that their engagement supports TCR-mediated signals, promoting effector responses including enhanced cytokine production, proliferation and survival. This dissertation builds on this work to clearly demonstrate that MyD88-dependent signals in T cells play a critical role in supporting *in vivo* T cell-responses against a viral pathogen. While MyD88 expression in T cells during LCMV infection appears to be dispensable for initial activation, proliferation, and differentiation into fate committed subsets of effectors, it is required for the survival and accumulation of effector T cells during the phase of antigen driven proliferation. These findings suggest a model (Figure 5.1) in which MyD88 plays an important, indirect role in promoting initial T cell activation through APCs in the lymph nodes but also acts directly in effector T cells at the site of infection, to provide signals that support the survival and clonal expansion. Thus MyD88 may serve as link that allows T cells to directly respond to inflammatory stimuli that deliver pro-survival signals during viral infection. At this point, the nature of the inflammatory stimuli that link MyD88 to T cell survival remain unclear and raises the possibility that the importance of MyD88 may be related to functions outside of it traditional role in TLR signaling during LCMV infection. Overall, by

broadening the importance of MyD88 in T cells, our findings support a shift in the dogma that restricts the role MyD88 to cells of the innate immune system and may have significant implications for understanding the signals that control T cell survival during inflammatory immune responses.

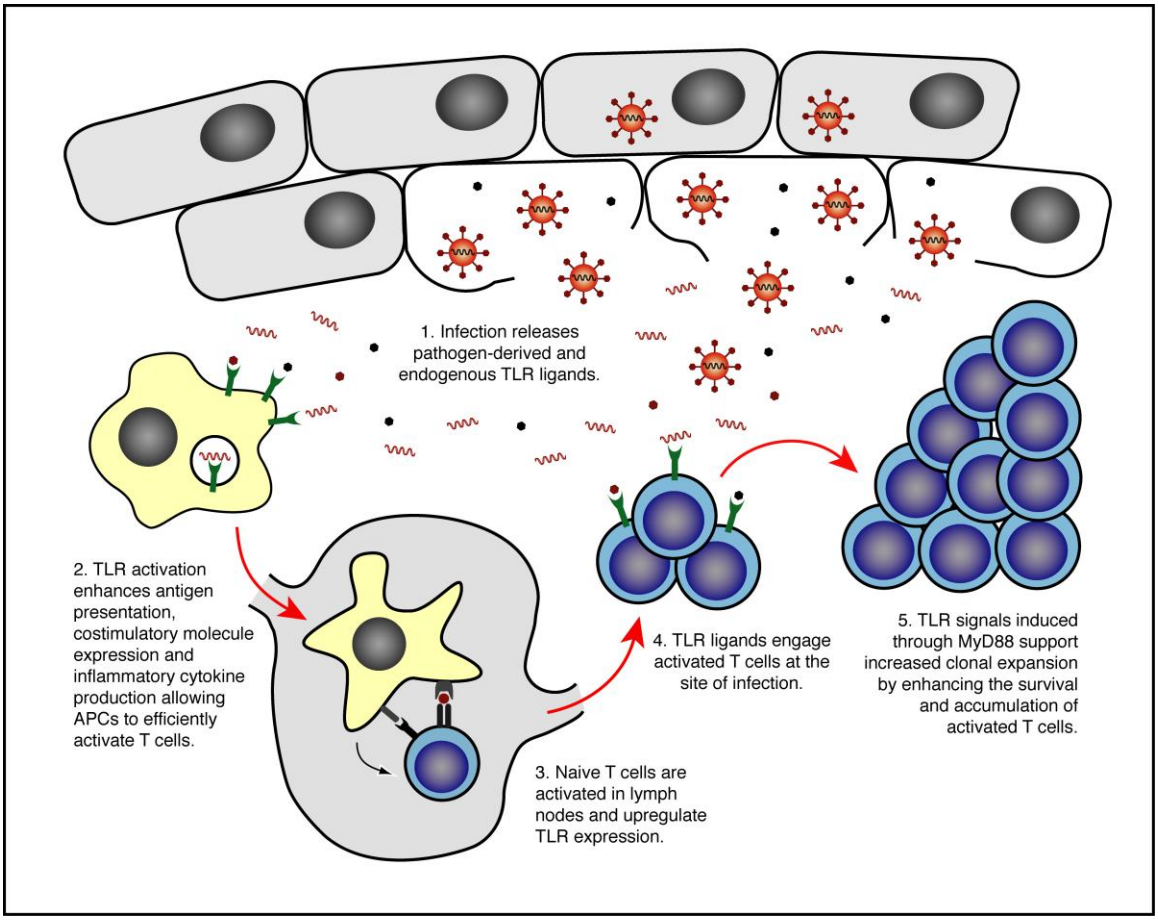


Figure 5.1. Model for the direct and indirect contributions of MyD88 to T cell responses during infection.

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