THE ROLE OF CD8 T CELLS IN THE PATHOGENESIS OF CUTANEOUS LEISHMANIASIS

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DEDICATION

I would like to dedicate this dissertation to all of the great mentors that have played a part in getting me to this point in my life, both personally and professionally, especially Kari Kromko, Dr. Ken Field, and Dr. Phillip Scott.

"Education is not the filling of a pail, but the lighting of a fire."

-William Butler Yeats

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ABSTRACT

THE ROLE OF CD8 T CELLS IN THE PATHOGENESIS OF CUTANEOUS LEISHMANIASIS

Erika J. Crosby

Phillip Scott, Ph.D.

Cutaneous leishmaniasis is a significant global health concern with more than 350 million people worldwide at risk of infection. One of the most interesting and confounding aspects of leishmaniasis is the broad spectrum of disease severity seen in patients. The role of CD8 T cells during leishmaniasis has been controversial, but is currently thought to entail the production of IFN-γ to polarize CD4 T cells towards a protective Th1 type immune response. However, analysis of CD8 T cells in human lesions does not demonstrate significant IFN-γ production by these cells. Additionally, CD8 T cells taken from patient lesions are responsive to a number of other pathogens, indicating significant infiltration of bystander CD8 T cells into the lesions during natural infection. Given this, we hypothesized that a patient's immunologic history may have a profound effect on the disease progression of *Leishmania* infection. To test this we examined the functionality of CD8 T cells, both antigen specific and bystander cells generated in response to other pathogens, at the site of infection with *L. major*. We show that CD8 T cells in the skin adopt a cytotoxic phenotype and express high levels of gzmB but little IFN-γ. To evaluate the potential role of bystander cells in the lesions during *L. major* infection, we utilized two distinct infection scenarios: a prior viral or bacterial infection that is cleared before challenge with *L. major* and a direct coinfection with a virus at the peak of lesion formation. In both cases, bystander cells exacerbated the disease course of *L. major*. In the case of a previous infection, this immunopathology was not associated with any change in parasite control. Whereas, in the case of direct coinfection the immunopathology was associated with an increased

parasite burden in both the skin and DLN. Strikingly, regardless of the timing of the heterologous infection, the immunopathology was mediated in a CD8 T cell, NKG2D dependent manner. This work identifies NKG2D and its ligands as potential therapeutic targets in leishmaniasis and has broad implications for the potential role of this pathway in other infections where excessive tissue damage and immunopathology are observed.

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Chapter 1 Introduction

1.1 Leishmaniasis

Leishmaniasis is a significant neglected tropical disease with more than 350 million people in 98 countries worldwide at risk of infection. There are an estimated 1.3 million new infections and 20,000 to 30,000 deaths occurring as the result of *Leishmania* infection every year (Alvar et al., 2012; World Health Organization, 2014). It is caused by infection with one of several different species of the protozoan parasite that are transmitted through the bite of an infected sandfly. There are three main forms of the disease: visceral, cutaneous, and mucocutaneous, with cutaneous infection accounting for the majority of total infections (World Health Organization, 2014). Even though cutaneous infection is typically not life threatening, healing of cutaneous lesions is still associated with the development of disfiguring scars on exposed skin surfaces. Additionally, it is a chronic infection that persists even after overt disease has resolved. Under conditions of severe malnutrition or immune suppression, reactivation can occur (Okwor and Uzonna, 2013). Another poorly understood aspect of disease is the diverse spectrum of clinical severity seen even among patients infected with the same species of *Leishmania*. Despite the significant disease burden, there is currently no vaccine available and treatment options are ineffective and costly, making leishmaniasis an important area of continued research (Kedzierski, 2010; Romero et al., 2001; Singh and Sundar, 2012).

As with any vector borne disease, understanding the distribution and biology of the vector has important implications for understanding the disease. The sandfly has a lifespan of less than 30 days and can only travel short distances; therefore, endemic regions are constrained to areas near larval breeding sites (Maroli et al., 2013). Man-

made environmental changes in the natural habitats of these vectors, the spread of immunosuppressive conditions in humans, deforestation, the establishment of new settlements and cities, and the consequences of widespread war and conflict in endemic regions have all contributed to the recent spread of infections into countries that historically have not had endemic disease (Antoniou et al., 2013). It has recently been appreciated that sandflies are not only the vector, but also are directly involved in the pathogenesis and immune response to *Leishmania*. Humans and rodents repeatedly exposed to the bites of uninfected sandflies develop IgG antibodies and cellular responses against salivary components (Rohousova et al., 2005). However this immune activation does not appear to be protective against infection, but rather the presence of sandfly saliva components in an experimental setting increase the infectivity of the parasites (Rohousova and Volf, 2006). Future studies are aimed at understanding if there is a role for sandfly saliva in the establishment of protective immunity and if it should be included in future vaccine strategies.

In addition to humans, rodents and dogs can serve as secondary reservoirs for the parasite (Maroli et al., 2013). Rodents being a natural host for *Leishmania* make them an ideal model system to study infection in the laboratory. In 1973, infection of various inbred mouse strains revealed differences in the sensitivity of different strains to *Leishmania* infection (Kellina, 1973). This observation began a series of studies aimed at understanding the immunological basis for this predisposition for susceptibility or resistance to *Leishmania* infection. Ultimately, it was determined that susceptibility correlates with the dominance of an IL-4 driven T_H2 response whereas an IL-12/IFN-γ driven T_H1 response promotes protection and parasite control (Heinzel et al., 1989; Heinzel et al., 1993; Mosmann et al., 1986; Nasseri and Modabber, 1979; Scott et al., 1988; Sypek et al., 1993). More recent work has challenged the simplicity of this model,

and while the basic paradigm still holds true, we now understand that there is more to susceptibility and resistance than just the T_H1/T_H2 dichotomy.

Leishmania parasites have a dimorphic life cycle: extracellular, flagellated promastigotes divide and mature within the midgut of sandflies and intracellular amastigotes reside and multiply within mammalian phagocytic cells. Immediately following inoculation into the skin, parasites are rapidly phagocytosed by resident dermal macrophages and dermal dendritic cells (DCs) (Locksley et al., 1988; Ng et al., 2008). Neutrophils also rapidly infiltrate the infected skin and phagocytose promastigotes (Laskay et al., 2003; Peters et al., 2008). Similar to *Mycobacterium* or *Salmonella*, once in the mammalian host, *Leishmania* survive inside the phagolysosome of the infected cell. In fact, the acidic environment present in the phagolysosome is important for the maturation of the parasite from promastigote to amastigote (Meresse et al., 1999; Russell et al., 1992). After several asexual divisions, the amastigotes rupture the host cell and are rapidly taken up by other phagocytes, mainly macrophages (Scott et al., 2004). Control of the parasite is mediated through activation of infected macrophages by IFN-γ to express nitric oxide. This requirement for IFN-γ is ultimately why resistance is characterized by a T_H1 type immune response and why examining the source of this IFN-γ has been the focus of so much work.

CD4 T cells are critically important for the control of *Leishmania* infections because they serve as the main source of IFN-γ (Scott, 1991). Mice that are deficient in CD4 T cells, IL-12p40, IFN-γ, or iNOS all fail to control parasite replication (Belkaid et al., 2000; Mattner et al., 1996; Swihart et al., 1995). Similarly, mouse strains that would typically be susceptible to infection can be treated with IL-12, develop a TH1 response, and become resistant to infection (Heinzel et al., 1993; Sypek et al., 1993). CD4 T cells are

known to be required not only for control of the primary infection, but are also required for long lived secondary immunity. Despite this, the establishment and maintenance of *Leishmania*-specific CD4 memory cells is not well understood. Previous work has demonstrated that the maintenance of protective immunity is dependent on low level persistence of parasite, but central memory CD4 T cells can persist in the absence of parasite and can provide some level of protection when transferred into naïve hosts (Belkaid et al., 2002; Scott et al., 2004). However, transfer of central memory CD4 T cells alone cannot offer the same level of protection as seen in an immune animal, suggesting that other elements of the immune response or parasite persistence are also playing a role; this is an area of ongoing investigation in our lab.

1.2 CD8 T cells and Leishmaniasis

While the role of CD4 T cells has long been established, the role of CD8 T cells during *Leishmania* infection has been much more controversial. Initial studies suggested that CD8 T cells were important in control of infection in both susceptible and resistant strains, demonstrating that depletion of CD8 T cells during infection made lesions larger and slower to heal (Titus et al., 1987). Similarly, in the susceptible BALB/c strain, if CD4 T cells were depleted, the remaining CD8 T cells were able to control parasite replication and confer resistance (Hill et al., 1989; Muller et al., 1991). However, later studies ruled out a role for CD8 T cells during primary infection, showing that CD8 deficient or β2-microglobulin deficient mice had no change in the course of *Leishmania* infection (Huber et al., 1998; Overath and Harbecke, 1993; Wang et al., 1993). Mice deficient in granzyme A, granzyme A and B, or perforin, the major cytotoxic granule components, also had no change in lesion resolution or parasite control (Conceicao-Silva et al., 1998; Eisert et al., 2002). Despite their dispensability during the primary response, it was

agreed upon that CD8 T cells were crucial for protective secondary responses through their production of IFN-γ (Colmenares et al., 2003; Muller et al., 1991; Muller et al., 1993).

All of these studies examining the role of CD8 T cells had utilized a high dose, footpad infection. Strikingly, when CD8 deficient mice were challenged with a lower dose, intradermal infection, they failed to control parasite growth (Belkaid et al., 2002). Further studies from our lab went on to show that at this lower dose CD8 T cells were a crucial source of IFN-γ early during infection to establish the protective T_H1 CD4 T cell response (Uzonna et al., 2004). Following infection with both doses, there is a burst of IL-4 production, but this burst is much larger following infection with a low dose of parasite. IFN-γ production by CD8 T cells was required to down-regulate this IL-4 response following low dose but not high dose infection. In the low dose model of infection, CD8 T cells were shown not only to be protective, but also to mediate pathology (Belkaid et al., 2002). Our lab has recently used a similar model and demonstrated that cytotoxicity, and not cytokine production, is responsible for the parasite independent pathology caused by these CD8 T cells (Novais et al., 2013). We also visualized direct killing of infected cells by CD8 T cells taken from lesions; interestingly in vitro evidence suggests that killing of an infected cell by a CD8 T cell does not result in the killing of the parasite (Smith et al., 1991). Together these results underscore an unappreciated role for CD8 T cells in not only the establishment of protective immunity to *Leishmania* infection, but also a potential role in the pathology of the disease as well.

In humans, CD8 T cells have also been shown to participate during *Leishmania* infections (Da-Cruz et al., 1994). Analysis of peripheral blood mononuclear cells (PMBCs) taken from patients with active or healed cutaneous leishmaniasis exhibited

significant cytotoxicity against *L. major* infected autologous macrophages (Bousoffara et al., 2004). Furthermore, cell lysates of parasite stimulated PBMCs from patients with active infections were analyzed for gzmB activity as a surrogate marker of a cytotoxic response and 62.5% of them had increased gzmB levels compared to healthy controls (Bousoffara et al., 2004). Histological analysis of early and late stage lesions from patients revealed a fivefold increase in the number of CD8 T cells present in late, more severe lesions compared to smaller, early lesions (Faria et al., 2009). Interestingly, this analysis further demonstrated an increase in the levels of granzyme A within CD8 T cells as lesions progressed from early to late, however no significant production of IFN-γ by CD8 T cells was observed. Similar analysis of lesions was recently completed by a second group who also concluded that recruitment of granzyme expressing CD8 T cells was associated with lesion progression and positively correlated with lesion severity (Dantas et al., 2013; Santos et al., 2013).

Transcriptional analysis of lesional skin revealed that some of the most highly expressed genes compared to normal skin were components of cytotoxic granules (Novais et al., 2013). Overall, data from patients confirms a role for CD8 T cells during *Leishmania* infection, but seems not to point to cytokine production and protection but rather cytotoxicity and pathogenicity as their major function. A better understanding of the role and regulation of CD8 T cells during infection is required for the generation of effective vaccines or novel therapeutic interventions.

1.3 Coinfections

Disease caused by persistent infections, like *Leishmania*, impact more than a third of the world's population. In addition to posing a significant health risk, chronic infections have also been shown to impact the ability to appropriately respond to other infections or

vaccinations (reviewed in Stelekati and Wherry, 2012). In some cases, chronic infections have been associated with an increased ability to control other infections, but the vast majority of evidence indicates an increased susceptibility to secondary infections and a reduced vaccination efficacy. A better understanding of the specific immunological effects of coinfections is necessary to design targeted therapeutics and effective vaccines for populations that suffer from chronic infections.

HIV infection represents a significant global health concern, and is complicated further by the high incidence of coinfections occurring in these immune compromised individuals. An estimated 4.5 million people are coinfected with HIV and Hepatitis C virus (HCV). This coinfection is associated with higher HCV persistence, faster progression of the disease and poor response to current treatments (Lambotin et al., 2012). Liver disease caused by Hepatitis B virus (HBV) is one of the leading causes of morbidity and morality in coinfected HIV patients (Iser and Sasadeusz, 2008). In 2010, an estimated 350,000 deaths were attributed to complications caused by tuberculosis coinfection in HIV infected individuals (Gray and Cohn, 2013). Together, these data suggest that a critical factor in preventing or treating global health threats like HIV will be addressing the complexities of immunological interactions during coinfections.

Another commonly occurring and well-studied coinfection is that of respiratory viruses and secondary bacterial infections. Autopsy case reports indicate that >90% of deaths during the 1918 influenza pandemic were likely cause by secondary infection with pneumococcal bacteria (Morens et al., 2008). Present day, on average approximately 6,000 people die of secondary bacterial infections following influenza in the United States every year (Centers for Disease Control and Prevention (CDC), 2010). Historically the mechanisms responsible for the viral/bacterial comorbidity have been

attributed to virus-induced lung tissue damage. However, the use of mouse models has more recently implicated a dysfunctional innate immune response for the enhanced susceptibility (reviewed in Metzger and Sun, 2013). Recent work from Medzhitov's group further implicates an impaired ability to tolerate tissue damage rather than failure to control bacterial infection (Jamieson et al., 2013). Ultimately, this century old observation of viral induced predisposition to bacterial infection is still not well understood and is an important ongoing area of research.

As was previously mentioned, *Leishmania* infection persists chronically, even after resolution of overt disease. Reactivation of *Leishmania* infection has become a significant problem, particularly in patients coinfected with HIV (Okwor and Uzonna, 2013). The first case of *Leishmania*/HIV coinfection was reported in 1985 and 35 countries have since reported incidences of coinfection (Alvar et al., 2008). Coinfection of cutaneous leishmaniasis and HIV is associated with an increased rate of leishmanial recurrence and a decreased responsiveness to therapy (Couppie et al., 2004). However, HIV is not the only coinfection shown to commonly occur with *Leishmania*. Intestinal helminth infection is also highly prevalent in regions that are endemic for leishmaniasis. Patients coinfected with *Leishmania* and an intestinal helminth demonstrated a healing time of nearly twice that of patients without coinfection (O'Neal et al., 2007). Despite this observation, early anti-helminthic treatment did not improve the clinical outcome of coinfected patients (Newlove et al., 2011). This highlights the need for a better basic understanding of how these pathogens, and the immune responses to these pathogens, impact one another.

Various experimental mouse models have been used to analyze the impact of other infections on the course of *Leishmania* disease. Infection with *Strongyloides ratti* prior to

infection with *L. major* had no effect on the disease course or parasite burden of either parasite (Kolbaum et al., 2011). Alternatively, infection with a different helminth, *Schistosoma mansoni (S. mansoni)*, prior to infection with *L. major* led to delayed resolution of footpad swelling and increased parasite burden, although the *L. major* infection was eventually controlled (La Flamme et al., 2002). The increased parasite burden was attributed to a defect in the production of NO by macrophages from *S. mansoni/ L. major* coinfected mice. In contrast, infection with *Toxoplasma gondii* (*T. gondii*) or *Listeria monocytogenes* prior to infection with *L. major* protected a susceptible strain of mice (Santiago et al., 1999; Tabbara et al., 2004). These coinfected mice had much lower parasite burdens and decreased tissue damage, which were associated with suppression of IL-4 and increased IFN-γ production in both cases. In summary, there are experimental examples of coinfections being protective, being pathologic, or having no effect on the disease course depending on the specific timing and infectious agents involved. This is likely the case in humans as well, when infectious histories are complicated and specific to each individual, and we hypothesize that it may be part of the explanation for the diversity seen in disease severity and duration among patients.

1.4 Heterologous immunity: cross-reactivity versus bystander T cells

One of the hallmarks of adaptive immunity is the establishment of pathogen specific memory responses that are maintained long term. Although this is a well-accepted attribute of an immune response, most experimental models studying these immune responses use pathogen free, 'naïve' mice. This is in contrast to humans who are continuously exposed to different pathogens and have populations of memory cells with the potential to respond to or cross-react with new infectious agents. The existence of these memory populations forms the basis for heterologous immunity, or the ability of

memory cells to respond to and influence the outcome of subsequent, unrelated infections. There are two main ways that cells are able to do this: bystander activation or TCR cross-reactivity. Bystander activation occurs when cells respond to inflammatory signals in an antigen independent manner. TCR cross-reactivity occurs when cells respond to an epitope that is distinct, yet similar to the epitope for which they were initially primed. However, the existence and relevance of bystander T cell activation has long been a topic of debate in the field. Initially because of its magnitude, the massive expansion of T cells observed during viral infection was thought to be accounted for in large part by bystander activation. However, tetramer analysis revealed that >80% of the activated T cells present at the peak of infection are actually antigen specific (Butz and Bevan, 1998; Masopust et al., 2007; Murali-Krishna et al., 1998; Tough and Sprent, 1998). The concept of bystander T cell activation, independent of specific T cell receptor (TCR) stimulation, challenges one of the fundamental tenants of the immune response-specificity. It is widely acknowledged that stringent regulation of every step of the immune response is required to avoid unnecessary immunopathology, lympho-proliferative disorders, and autoimmunity. Therefore, the activation of a T cell independent of its TCR seemingly bypasses the intricate regulatory system set up to protect the host from abhorrent immune activation. Despite the apparent dangers of bystander activation, many experimental infection models show bystander CD8 T cell activation as an important mechanism of early protection (Berg et al., 2003; Chu et al., 2013; Soudja et al., 2012). In light of this, we will discuss what is known about bystander T cell activation during infection and inflammation, how it differs from TCR cross-reactivity, and evidence for both scenarios in humans.

Bystander activation can be mediated by inflammatory cytokines. Type I IFNs or IL-12 and IL-18 that are given exogenously, or produced in response to infection or

inflammation, can induce the proliferation and activation of memory phenotype ($CD44^{hi}$) CD8+ T cells (Beadling and Slifka, 2005; Berg et al., 2003; Kamath et al., 2005; Kambayashi et al., 2003; Raué et al., 2004; Raué et al., 2013; Tough et al., 1996). Direct injection of IFN-α/β can cause proliferation of memory phenotype CD8 T cells, even in β-2-microglobulin deficient animals, demonstrating the TCR-independent nature of this activation (Tough et al., 1996). This activation in response to inflammatory cytokines is functionally relevant, as bystander CD8 T cells have been shown to provide increased protection from an unrelated respiratory virus through expression of gzmB in an IFN-α receptor dependent manner (Kohlmeier et al., 2010). Other work with *Listeria* infection demonstrates that type I interferon dependent gzmB induction is modulated through the secondary production of IL-15 by monocytes (Soudja et al., 2012). In this model, IFN-γ production by bystander CD8 T cells seemed to be IL-12/18 dependent, whereas gzmB expression was mediated by IL-15. Additionally, immunotherapeutic regimens for cancer using high dose IL-2 treatment have provided evidence of antigen-independent, cytokine driven activation of CD8 T cells that can result in significant anti-tumor effects (Tietze et al., 2012). Some recent in vitro work really demonstrates the remarkable array of cytokines and cytokine combinations that memory CD8 T cells can mount distinct responses to, highlighting the complexity and impact of a specific cytokine milieu (Freeman et al., 2012).

A separate consequence of a heterologous immune response is TCR cross-reactivity. The notion of specificity in regards to the TCR is a somewhat misleading notion, as each TCR has the ability to bind many different peptides, albeit with varying affinities (Reiser et al., 2003). It is also not completely surprising that there is some cross-reactivity observed within responses, especially those directed at evolutionarily conserved proteins within groups of viruses or bacteria (Boon et al., 2004; Effros et al., 1977;

Mongkolsapaya et al., 2003). A cross-reactive TCR response is fundamentally a different type of immune response than bystander activation that is caused by cytokines; it signals through different pathways and is characterized by distinct functional outcomes. An elegant mouse model was recently created that can distinguish signals through the TCR from activation induced by inflammatory cues; cells receiving a TCR signal begin to express GFP (Moran et al., 2011). The expression of GFP is strictly dependent on a TCR stimulus and is directly correlated with the signal strength of the TCR, making it a powerful tool to distinguish bystander activation from cross-reactivity. Prior to this, most cross-reactive responses were identified by the observable changes to the immunodominance hierarchy of the original memory T cell pool. TCR cross-reactivity often results in the preferential expansion of a cross-reactive sub-dominant T cell epitopes, often leading to an alteration in the hierarchy and a narrowing of the remaining memory T cell responses from the initial infection (Reviewed in Haanen et al., 1999; Selin et al., 2006). The use of tetramer technology has allowed for some specific cross-reactive epitopes to be identified; however, most are still not known. Ultimately, being able to distinguish cross-reactivity from bystander activation will be important when attempting to modulate these responses therapeutically.

Similar to bystander activation, cross-reactivity can be protective against subsequent infections. A population of memory CD8 T cells generated by lymphocytic choriomeningitis virus (LCMV) infection expanded and became activated in response to infection with vaccinia virus (VV), resulting in significantly enhanced viral clearance and decreased mortality following an otherwise lethal VV infection (Chen et al., 2001). This group has further gone on to show the exact cross-reactive epitopes responsible for this protective immunity (Cornberg et al., 2010). Other studies demonstrate that while cross-reactivity can be protective, it can also be pathologic. While previous LCMV infection is

protective against VV, it inhibits the clearance of Respiratory Syncytial Virus (RSV) (Ostler et al., 2003). Similarly, a history of influenza A (Flu) infection protects against VV infection, but inhibits clearance of murine cytomegalovirus (MCMV) and LCMV (Chen et al., 2003). Another major pathological occurrence during a cross-reactive heterologous immune response is the development of auto- and allo-reactivity. The induction of autoimmune disease or transplant rejection often occurs during heterologous immune responses because of molecular mimicry that allows the 'licensing' of an autoreactive T cell response during the inflammatory setting of infection. CD8 T cells that display allospecific cytotoxic activity have been identified following LCMV, Pichinde virus (PV), VV, and MCMV infection in mice (Burrows, 1999; Yang, 1989; Strang, 1987; Tomkinson, 1989). Similarly, a mouse model of autoimmune keratitis is induced by herpes simplex virus-type 1 (HSV-1) infection because T cells specific for the HSV-1 UL6 protein cross-react with a corneal antigen (Zhao et al., 1998). To fully understand the important role that memory T cells play in balancing the induction of protective immunity versus immunopathology, the field needs to better understand the interactions between heterologous immune responses, whether they are cross-reactive, bystander activation, or more likely a combination of both.

Instances of cross-reactivity and bystander activation have been observed in humans. A recent longitudinal study was performed to examine the impact of primary Epstein-Barr virus (EBV) infection on preexisting memory CD8 T cells specific for Flu and cytomegalovirus (CMV) (Odumade et al., 2012). During natural primary EBV infection, both flu and CMV specific CD8 T cells became activated and expressed gzmB but showed no signs of expansion, implicating bystander activation and not cross-reactivity. In contrast, specific cross-reactive responses have been identified in humans between flu and HCV (Wedemeyer et al., 2001). Interestingly, analysis of T cell responses

revealed that patients with an unusually narrow HCV response that included an over representation of these cross-reactive flu epitopes presented with a severe fulminant, necrotizing hepatitis (Urbani et al., 2005). Overall, cross-reactivity in humans has been linked to the induction of immunopathology, not necessarily because it is the most common manifestation, but because this outcome is easier to measure than increased resistance to disease. What is increasingly evident is that our understanding of how immune responses to heterologous pathogens impact one another during natural infections is severely lacking. As personalized medicine grows in popularity, an individual's immunologic history should likely be a critical component of a successful treatment plan.

1.5 NKG2D and CD8 T cells

NKG2D is an activating receptor that is expressed on all NK cells, most NKT cells and subpopulations of γδ T cells (Raulet, 2003). In humans, all CD8 T cells also express NKG2D, but in mice only a subset of activated and memory CD8 T cells do (Diefenbach et al., 2000; Girardi et al., 2001). NKG2D is a transmembrane C-type lectin-like receptor that is encoded by the gene KLRK1 (killer cell lectin-like receptor subfamily K member 1) (Guerra et al., 2008). Unlike other members of the NKG2 family, it forms a homodimer and has no inhibitory component (Champsaur and Lanier, 2010). In mice, there are two isoforms of NKG2D that are generated by alternative splicing, a long form (NKG2D-L) and a short form (NKG2D-S) (Diefenbach et al., 2002). These different isoforms recruit different adapter proteins; NKG2D-L associates exclusive with DAP10, whereas NKG2D-S can pair with either DAP10 or DAP12. Humans, however, only express the long form and therefore can only pair with DAP10 (Rosen et al., 2004). While DAP12 can bind NKG2D-S, deficiency in DAP10 results in complete loss of NKG2D signaling in

T cells, indicating that it is the crucial adaptor protein, even in mice (Groh et al., 2002; Karimi et al., 2005).

NKG2D recognizes a family of stress-induced ligands that are MHC class I protein homologs and new members of this ligand family continue to be discovered in both mice and humans. The first ligands to be described were MHC class I chain-related protein A (MICA) and B (MICB) that were expressed in the gastrointestinal epithelium (Groh et al., 1996). Subsequently another family of ligands that bound the human cytomegalovirus glycoprotein UL16 (ULBP16) was discovered (Cosman et al., 2001). The orthologs of these genes in mice were identified as the retinoic acid early inducible cDNA clone-1 (Rae1) and were subsequently shown to also be NKG2D ligands (Nomura et al., 1994; Zou et al., 1996). Presently there are five known members of the Rae1 family: Rae1α, Rae1β, Rae1γ, Rae1δ, and Rae1ε, all of which are differentially expressed in different tissues and mouse strains but are highly homologous to one another (>85% identity). Another family of NKG2D ligands in mice are Rae1 related proteins, histocompatibility antigen 60 a (H60a), H60b and H60c (Malarkannan et al., 1998; Takada et al., 2008). H60c has been shown to be especially highly expressed in the epidermis during wound repair (Yoshida et al., 2012). The final NKG2D ligand to be discovered in mice is a murine ULBP-16-like transcript 1 (MULT1), which is highly similar to the human ULBP proteins (Carayannopoulos et al., 2002). These ligands are upregulated under conditions of cellular stress, although ligand transcripts and occasionally protein can be found in normal cells (Takada et al., 2008). There is also experimental evidence that these ligands can be posttranslationally regulated, making RNA message levels an unreliable readout of protein expression but allowing the cell to quickly respond to environmental triggers (Nice et al., 2009). Overall, these ligands act as molecular

sensors, triggered by infection, DNA damage, and transformation, to activate cells of the immune system.

While NKG2D expression and function on NK cells has been well described, it was much more recently that its role on CD8 T cells has been investigated. In mice, it is inducibly expressed by a subset of activated CD8 T cells. After 5 days of in vitro stimulation of splenocytes with anti-TCRβ nearly all of the CD8 T cells had upregulated NKG2D, whereas none of the CD4 T cells had (Ehrlich et al., 2005; Jamieson et al., 2002). Similarly, the majority of transgenic OTI CD8 T cells that were stimulated with ovalbumin peptide in vitro had upregulated NKG2D by day 5, but a small population remained NKG2D negative (Ehrlich et al., 2005; Jamieson et al., 2002). Additionally, following infection of mice with LCMV, all GP33 tetramer positive cells had expressed NKG2D by 9 days post infection and expression remained high in these cells for at least 8 months following infection (Jamieson et al., 2002). Another group showed that DUC18 transgenic CD8 T cells (specific for an ERK2 kinase mutant found in many fibrosarcomas) upregulated NKG2D upon in vitro stimulation, but that after subsequent rounds of stimulation the expression steadily decreased (Markiewicz et al., 2005). Despite these reports, not all CD44 positive CD8 T cells in a mouse express NKG2D, thus the exact requirements for expression and maintenance of NKG2D expression by CD8 T cells remains an active area of study.

Similar to studies of the expression of NKG2D by CD8 T cells, much of the work on its exact functional role in CD8 T cells is contradictory and seems to be rather context dependent. Using a CD8 T cell line that expresses NKG2D or an in vitro activated transgenic CD8 T cell, it was shown that crosslinking NKG2D alone did not induce a response, but it did have a costimulatory effect on proliferation, cytotoxicity, IFN-γ

production and calcium flux similar to CD28 when crosslinking occurred in conjunction with TCR stimulation (Jamieson et al., 2002; Markiewicz et al., 2005). However, another study using in vitro activated OTI cells reported that NKG2D did not costimulate in vitro proliferation or cytotoxicity of these cells (Ehrlich et al., 2005). One interesting difference seen in vitro between NKG2D and CD28 was that NKG2D engagement with its ligand could induce the formation of an immune synapse in the absence of cognate antigen while CD28 did not (Markiewicz et al., 2005). While classically the role of NKG2D on CD8 T cells is thought to be costimulatory in nature, growing evidence points to a more NK cell like functioning of this receptor in a TCR independent manner. Using human CD8 T cells that were subsequently activated in vitro, NKG2D and DAP10 dependent but MHC class I independent lysis of target cells has been demonstrated (Verneris et al., 2004). Treatment of mice with a cytokine based anti-CD40/IL-2 cancer immunotherapy expanded a population of NKG2D expressing memory CD8 T cells that were broadly cytotoxic against tumor cells (Tietze et al., 2012). Blockade of NKG2D during treatment resulted in significant decreases in the observed antitumor effects. Strikingly, treatment of TCR transgenic mice bearing non-antigen expressing tumors still resulted in significant antitumor cytotoxicity, highlighting the antigen independence of the antitumor immunity. Overall, in vitro studies have been somewhat inconclusive about the exact costimulatory function of NKG2D on CD8 T cells and it is likely highly context dependent. In contrast, both in vitro and in vivo studies have supported a TCR independent role for NKG2D in cytotoxicity by CD8 T cells. This innate-like functioning of memory CD8 T cells is a provocative concept, particularly when considered in the context of human immunity and therapies, and requires additional basic questions to be answered about its regulation, expression, and function before it can be utilized or manipulated therapeutically.

1.6 Summary

Previous studies of CD8 T cells during Leishmania infection have identified a role for these cells in producing IFN-γ, but patient data seems to indicate that this is not the whole story. In fact, mounting evidence suggests that CD8 T cells may be responsible for the severe immunopathology that occurs (Faria et al., 2009; Novais et al., 2013; Santos et al., 2013). Here we will evaluate the function of both antigen specific and non-antigen specific CD8 T cells in the skin during *L. major* infection. We know that non-*Leishmania* specific CD8 T cells are recruited into the lesions of patients, therefore we will utilize mice that have had previous, unrelated infections to examine the impact of unrelated memory cells on disease progression (Da-Cruz et al., 2010). The long disease course and persistent nature of *Leishmania* infection means that patients are at high risk for coinfection with other pathogens that likely will impact their immune response to *Leishmania*. Given this, we will also employ a model of coinfection occurring after the establishment of infection with *L. major*. In both cases we will examine the expression and function of NKG2D on CD8 T cells, with particular interest in its role in inducing pathology. The goal of these studies is to develop a better understanding of the mechanisms that induce immunopathology during leishmaniasis with the hope of creating effective, more specific treatments in the future.

Chapter 2 CD8 T cells in *Leishmania* infected skin have a cytotoxic phenotype

2.1 Abstract

Cutaneous leishmaniasis is a significant global health concern with more than 350 million people in 98 countries worldwide at risk of infection. Despite this significant disease burden, there are no vaccines available and treatment options are expensive and ineffective. Mouse models have provided insight into what a protective immune response to *Leishmania* infection qualitatively looks like, but have failed to translate this understanding into new treatments or vaccination strategies. The role of CD8 T cells in a protective immune response has been controversial, but is currently thought to entail the production of IFN-γ that is crucial to polarize CD4 T cells towards a protective Th1 type immune response. However, analysis of CD8 T cells in human lesions does not demonstrate significant IFN-γ production by these cells. Given this, we examined the functionality of CD8 T cells in the skin at the site of infection with *L. major*. We show that while CD8 T cells in the DLN can produce large amounts of IFN-γ, the CD8 T cells in the skin adopt a cytotoxic phenotype and express high levels of gzmB but little to no IFN-γ. By utilizing transfer experiments, we further show that gzmB is not only expressed by antigen specific cells, but is also induced in bystander, non-*Leishmania* specific CD8 T cells. This expression of gzmB independent of TCR signals is partially dependent on IL-15 production in the lesion. This work highlights an unappreciated function for CD8 T cells in the skin during *Leishmania* infection and underscores the impact of inflammatory signals in the lesion on this function.

2.2 Introduction

Leishmania is an intracellular protozoan parasite that is transmitted by the bite of an infected sandfly and causes a diverse clinical spectrum of disease severity (Sacks and Noben-Trauth, 2002). Cutaneous leishmaniasis can range from subclinical infections, to self resolving lesions, to chronic disseminated disease (Farah and Malak, 1971; Murray et al., 2005; Pearson and Sousa, 1996). A mouse model of cutaneous infection has demonstrated that protective immunity is primarily dependent upon the development of a strong Th1 CD4 T cell response, which leads to the production of IFN-γ that activates macrophages to kill the parasites (Kaye and Scott, 2011). It was long questioned whether CD8 T cells played any role in controlling a primary infection, as some studies showed no change in parasite burden or disease severity in the absence of CD8 T cells, while others demonstrated the ability of CD8 T cells to control parasite replication (Hill et al., 1989; Huber et al., 1998; Muller et al., 1991; Overath and Harbecke, 1993; Titus et al., 1987; Wang et al., 1993). They were, however, known to be critically important for protection from a secondary challenge (Colmenares et al., 2003; Muller et al., 1993). Recently it has been shown that CD8 T cells do play a protective role during primary infection, but the requirement for the CD8 T cells seems to be dependent on the infectious dose of parasite used (Belkaid et al., 2002; Uzonna et al., 2004).

Given the critical role for IFN-γ during *Leishmania* infection, much work has focused on the ability of CD8 T cells to produce IFN-γ. Several studies have demonstrated that early production of IFN-γ by CD8 T cells in the DLN is important to shift the immune response towards a protective Th1 response, particularly at low doses of infection (Belkaid et al., 2002; Uzonna et al., 2004). A role for cytotoxicity by CD8 T cells seemed unlikely given that infection of granzyme deficient mice had no effect on parasite burden

or disease progression (Eisert et al., 2002). Additionally, in vitro experiments demonstrated that lysis of infected macrophages by CD8 T cells did not result in killing of the parasites (Smith et al., 1991). However, protective vaccination of mice with a leishmanial antigen was abrogated in mice deficient in perforin, implicating a role for cytotoxicity in the protective immunity observed in this system (Colmenares et al., 2003). Conversely, recent work from our lab has implicated cytotoxicity by CD8 T cells in the induction of immunopathology and metastasis of parasites without any decrease in parasite burden (Novais et al., 2013). This pathology was also dependent on perforin expression by the CD8 T cells. Analysis of peripheral blood mononuclear cells from patients with active cutaneous leishmaniasis revealed increased granzyme B (gzmB) activity and significant cytotoxicity against *L. major* infected autologous macrophages (Bousoffara et al., 2004). Histological analysis of lesions from patients revealed a five-fold increase in the number of CD8 T cells present in late lesions compared to early lesions. Interestingly, the CD8 T cells in the late or more severe lesions were expressing high levels of granzyme whereas most of the IFN-γ being produced in the lesion was coming from CD4 T cells, not CD8 T cells (Faria et al., 2009; Santos et al., 2013). Overall the data from patients seems to indicate that our understanding of CD8 T cell function during *Leishmania* infection from the mouse model is incomplete and warrants more careful analysis.

CD8 T cells are often considered to be polyfunctional in that they are able to both act as cytotoxic cells and produce a variety of inflammatory cytokines, but the heterogeneity of CD8 T cell responses is highly dependent upon the local inflammatory cytokine milieu (Berg and Forman, 2006; Freeman et al., 2012; Wirth et al., 2011). Thus, whether CD8 T cells produce IFN-γ or increase their cytolytic activity is partially dependent on the cytokines present. For example, the combination of IL-12 and IL-18 promotes IFN-γ

production by CD8 T cells, while IL-15 or type I IFNs promote increased cytolytic activity (Beadling and Slifka, 2005; Berg et al., 2003; Berg et al., 2002; Kambayashi et al., 2003; Kohlmeier et al., 2010; Lertmemongkolchai et al., 2001; Raué et al., 2004; Raué et al., 2013; Yoshimoto et al., 1998). Increased IFN-γ production by CD8 T cells would be expected to promote increased resistance to intracellular pathogens like *Leishmania* that are known to be sensitive to IFN-γ. In contrast, other cytokines like IL-15, fail to induce large amounts of IFN-γ, but rather induce an increase in the expression of gzmB (Liu et al., 2002; Soudja et al., 2012; Yajima et al., 2005). It is therefore important to consider the inflammatory milieu present within the skin during *Leishmania* infection to fully understand the consequences it has on CD8 T cell function, as well as the role cytokine production by CD8 T cells has on the inflammatory process.

Overall, while much has been done to define the role of CD8 T cells in the DLN during *Leishmania* infection, little work has been done to evaluate their role at the site of infection in the skin. Data from patients indicates that there is a strong correlation between the severity of the disease and the number of CD8 T cells within the lesions. Given this, we focused on defining the population of CD8 T cells present in the leishmanial lesion and evaluating both their functionality and role in disease progression. We found that CD8 T cells infiltrate into the lesion very early on during infection and have a cytolytic phenotype while not making much IFN-γ. We went on to show that the inflammatory environment within the skin during infection drives this CD8 phenotype, and that gzmB production by CD8 T cells is partially dependent on IL-15 production. Ultimately it will be important to understand more fully which specific inflammatory signals are cueing this cytotoxic CD8 phenotype to potentially target them therapeutically.

2.3 Materials and methods

Animals

Female C57BL/6 mice and B6-Ly5.2/Cr (CD45.1) (6 weeks old) were purchased from the National Cancer Institute (Fredericksburg, MD). RAG2/OT-I mice were purchased from Taconic Farms. Transgenic reporter mice that expressed Thy1.1 under the control of the IFN-γ promoter were obtained from Casey Weaver and maintained in our animal colony (Harrington et al., 2008). Animals were housed in a specific pathogen-free environment and tested negative for pathogens in routine screening. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01.

Parasite, virus, and bacterial infections

L. major parasites (Friedlin) or *L. major* expressing ovalbumin (*L. major*-OVA) were grown to the stationary phase in Schneider's Drosophila medium (Gibco) supplemented with 20% heat-inactivated FBS (Gibco) and 2 mM L-glutamine (Sigma) at 26°C (Prickett et al., 2006). Metacyclic promastigotes were isolated from 4-5 day old stationary cultures by density gradients (Späth and Beverley, 2001). Mice were infected with 10^3 or 2 x 10^6 metacyclic parasites injected intradermally into the ear. Lesion development was monitored weekly by taking measurements of ear thickness with digital calipers (Fisher Scientific). Parasite burden in lesion tissues was assessed using a limiting dilution assay as previously described (Scott et al., 2004). For viral infections, mice were infected with 2 x 10^5 PFU of LCMV Armstrong strain by i.p. injection.

T cell transfers

For CFSE dilution experiments, CD8 T cells from CD45.1 OTI-RAG mice were isolated from the spleen and labeled with 2.5 μM CFSE for 5 minutes. The reaction was quenched with fetal calf serum and $2x10^4$, $2x10^5$, or $2x10^6$ OTI cells were transferred intravenously into each mouse. Prior to transfer of memory CD8 T cells from LCMV immune mice, splenocytes were enriched for T cells using the Pan T cell Isolation Kit II according to the manufacturer's instructions (Miltenyi Biotec). Enriched fractions were analyzed by flow cytometry and 3-4 x 10^6 CD8 T cells were transferred.

Flow cytometry

For flow cytometry, cells were isolated from ears, draining lymph nodes, spleens or peripheral blood. For ears, dermal sheets were separated and incubated in incomplete IMDM+GlutaMAX (Gibco) containing 0.25 μg/mL of Liberase TL (Roche, Diagnostics Corp.) and 10 μg/mL DNase I (Sigma-Aldrich) for 90 minutes at 37°C. Ears, draining lymph nodes, and spleens were mechanically dissociated by smashing through a 40-μm cell strainer (Falcon) in PBS containing 0.05% BSA and 20 μM EDTA. Splenocytes were incubated for <1 minute with ACK lysing buffer (Lonza) to lyse red blood cells. When indicated, cells were incubated at 4 x 10^6 cells/ml with Brefeldin A (BFA, 3 μg/ml final concentration) (eBioscience) alone for 5 hours or with phorbol myristate acetate (PMA) (Sigma, 100 ng/ml final concentration) and ionomycin (Sigma, 1 μg/ml final concentration) and BFA for 4-6 hours before staining for flow cytometry. Fixable Aqua dye (Invitrogen) was added to assess cell viability. Cells were then incubated with FC block (anti-CD16/32, heat inactivated mouse sera and Rat IgG) followed by fluorochrome-conjugated antibodies for surface markers CD45, CD45.1, CD45.2, CD8β, CD4, CD44, and/or CD62L (all eBioscience) and were fixed with 2% paraformaldehyde (Electron Microscopy Sciences). For intracellular staining, cells were previously permeabilized with 0.2% of saponin buffer and stained for IFN-γ and/or gzmB

(eBioscience or Invitrogen). The data were collected using an LSR Fortessa flow cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star).

Histopaque

Peripheral blood was collected into a 4% sodium citrate solution. White blood cells were isolated by underlaying with Histopaque-1083 (Sigma) and spinning for 20 minutes at 400 x g at room temperature.

ELISAs

Supernatants were analyzed using a sandwich ELISA with paired monoclonal antibodies to detect IFN-γ (eBioscience). Cytokine concentrations were calculated from standard curves with detection limit of 0.03 ng/mL for IFN-γ. Granzyme B was analyzed by ELISA using a mouse granzyme B Duoset kit (R&D Systems).

Statistics

Results represent means ± SEM. Data were analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined using unpaired, one-tailed Student's *t* test with p values given as: *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$; ns $p > 0.05$. Results with a p value ≤ 0.05 were considered significant.

2.4 Results

CD8 T cells infiltrate into the skin early after infection with *L. major*

Given the controversy surrounding the role of CD8 T cells during *L. major* infection, we began by examining the T cell infiltration into the infected skin very early, at one week after infection. We found a striking increase in frequency of CD8 T cells present in *L. major* infected ear skin compared to naïve skin, which in specific pathogen free mice has almost no CD8 T cells (Figure 1A). This represents a greater than 30 fold increase in

the number of CD8 T cells whereas the CD4 T cell population only increased by 2 fold (Figure 1B). It is important to note that human skin contains large numbers of T cells, an important difference to consider when evaluating the recruitment, activation and function of T cells in this tissue (Clark et al., 2006).

Much work has been done to determine the number of parasites that are deposited in the skin following the bite of an infected sand fly, and there appears to be a rather dynamic range from 10 to 10^5 (Kimblin et al., 2008). It is not known what impact the number of parasites deposited by the sandfly has on the severity or duration of disease. This experiment was done using $2x10^6$ metacyclic parasites and the only other careful characterization of CD8 infiltration into *Leishmania* infected skin was done using a much lower infection dose. This group showed that following low dose infection, CD8 T cell infiltration does not occur until 4-6 weeks and is rather modest in comparison to the CD4 T cells infiltration (Belkaid et al., 2002). Given this difference, we performed a kinetic analysis of T cell infiltration into the skin during the first 3 weeks to compare recruitment following low dose or high dose *L. major* infection. Consistent with previously published data, there was no observable increase in the percent or number of CD8 T cells in the first 3 weeks following low dose infection, whereas CD4 T cells began to increase 2 weeks after infection (Figure 1C and 1D). During a high dose challenge, there was a dramatic increase in the frequency of CD8 T cells in the first week that was maintained (Figure 1E and 1F). The CD4 T cells increased by a much smaller proportion compared to CD8 T cells during the first week but continued to increase throughout the first 3 weeks (Figure 1E and 1F). The infiltration of both CD4 and CD8 T cells was much more robust following high dose infection than low dose infection (Figure 1D and 1F). This is consistent with the observation that at 21 days post high dose infection there were visible lesions on the skin, whereas at the same time point following low dose infection

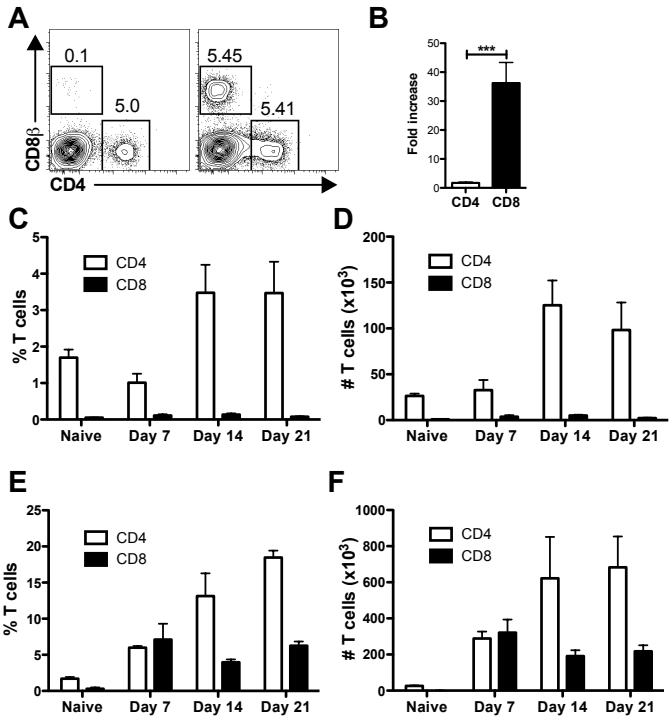

Figure 1. CD8 T cells are rapidly recruited to the skin following high dose *L. major* infection. Mice were infected with *L. major* or left uninfected and T cell infiltration into the skin was analyzed (A). The fold increase of CD4 or CD8 T cells in infected skin over naïve skin was calculated (B). The infiltration of CD4 and CD8 T cells into the infected skin after low dose (10^3; C-D) or high dose ($2x10^6$; E-F) infection with *L. major* was analyzed for the first 21 days. The percent of total cells in the skin (C and E) or absolute cell numbers (D and F) are shown. Data are representative of 5 independent experiments (A-B; n=3-5 mice per group) or 2 independent experiments (C-F; n=3-4 mice per group). Data shown in B are pooled from 5 experiments. Error bars represent the SEM.

there was no visible inflammation. In patients, CD8 T cells have been identified as a major cell population that is present within the lesions and increased numbers of CD8 T cells is correlated with increased disease severity (Dantas et al., 2013; Faria et al., 2009). Although our high dose of infection is still somewhat higher than what might be expected during natural infection, vaccines have been designed to deliver high doses of heat killed parasite or parasite antigens, many of which are given intradermally (Noazin et al., 2008). For these reasons, we chose to use high dose intradermal infection as a model to study the role of CD8 T cells within leishmanial lesions.

CD8 T cells in the skin express less IFN-γ than CD4 T cells

Our lab has previously shown a critical role for CD8 T cells to produce IFN-γ in the draining lymph node (DLN) early following *Leishmania* infection to help modulate CD4 T cells responses (Uzonna et al., 2004). In light of this, we examined IFN-γ production by CD4 and CD8 T cells at 1 week post infection in both the DLN and the infected skin. To get a sense of how cells were functioning in vivo we stained for IFN-γ after incubation with BFA alone and no other stimulation (Figure 2A). To measure their potential to make IFN-γ we also restimulated the cells with PMA/Ionomycin in the presence of BFA (Figure 2D). To better facilitate the comparison between T cells in the skin and DLN, we pregated on the activation marker CD44 when analyzing T cells in the DLN. With no restimulation, the only population of cells from the DLN or skin that were making detectable IFN-γ was CD4 T cells from the infected skin (Figure 2A-2C). When cells from the DLN were restimulated with PMA/Ionomycin, a population of both CD4 and CD8 T cells made IFN-γ, but CD8 T cells made significantly more than CD4 T cells (Figure 2D-2F). This is consistent with the previously demonstrated role for CD8 T cell derived IFN-γ in the DLN early after infection (Uzonna et al., 2004). In contrast, while both CD4 and CD8 T cells taken from the skin also made IFN-γ after restimulation, CD4 T cells made significantly more than CD8 T cells (Figure 2D). This was true by both percentage of CD4+ IFN-γ producers as well as on a per cell basis as reflected by the fluorescence intensity of IFN-γ (Figure 2E and 2F).

To further analyze IFN-γ production by these populations, we utilized an IFN-γ reporter mouse that expresses Thy1.1 on the surface of cells that are expressing IFN-γ (Harrington et al., 2008). The level of Thy1.1 protein on the surface of cells directly correlates with IFN-γ message levels. This allows us to detect cells that are actively transcribing IFN-γ without restimulation or intracellular staining. The Thy1.1 reporter

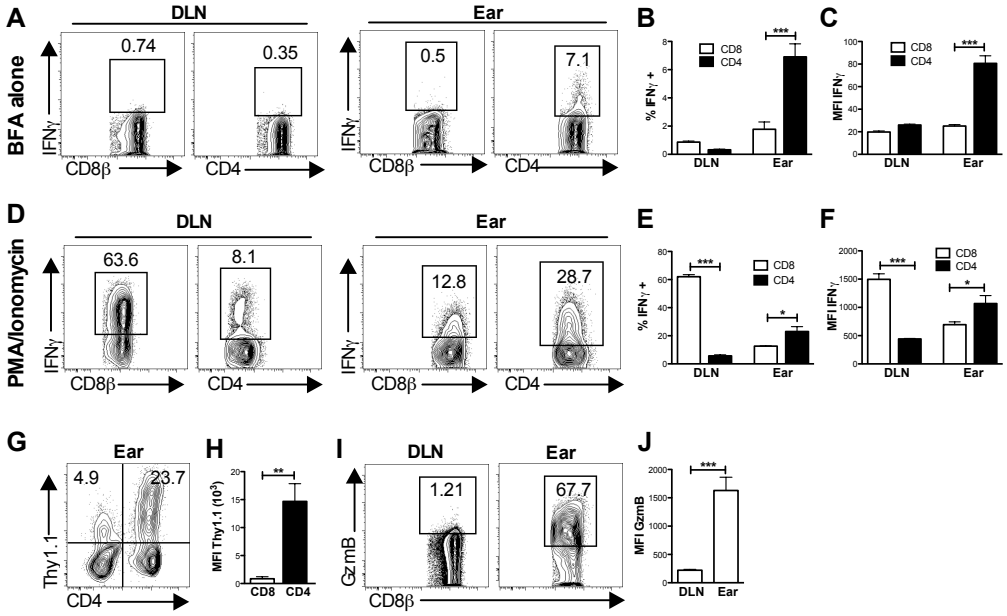

Figure 2. CD8 T cells in *L. major* infected skin express gzmB but not IFN-γ. B6 mice were infected with *L. major* and 1 week post infection the DLN and infected skin were harvested and incubated with BFA alone (A-C) or restimulated with PMA/ Ionomycin (D-F) and analyzed for IFN-γ production by flow cytometry. T cells in the DLN were pregated on $CD44^{hi}$. The percent (B and E) and MFI (C and F) of IFN-γ producing cells in the skin and DLN were calculated. Thy1.1 reporter mice were infected with *L. major* and 5 weeks post infection the infected skin was harvested and analyzed by flow cytometry (G). Plots were pregated on T cells. The MFI of Thy1.1 expression was calculated (H). B6 mice were infected with *L. major* and after 1 week the DLN and infected skin were harvested and incubated with BFA alone (I-J) and analyzed for gzmB production by flow cytometry. The MFI of gzmB expression was calculated (J). Data are representative of 3 independent experiments (A-C; D-F; I-J; n=3-5 mice per group) or 2 independent experiments (G-H; n=3-5 mice per group). Error bars represent the SEM.

mice were infected with *L. major* and at the peak of lesion formation, 5 weeks post infection, cells from the skin were analyzed directly ex vivo for Thy1.1 expression. Strikingly, significantly more CD4 T cells from the skin were expressing Thy1.1 than CD8 T cells (Figure 2G). The difference in expression levels of Thy1.1 between CD4 and CD8 T cells was also noteworthy, with CD4 T cells expressing much higher levels than CD8 T cells (Figure 2H). These results further highlight that CD8 T cells in the skin do not appear to be producing large amounts of IFN-γ; rather CD4 T cells are the main IFN-γ producers in the skin.

CD8 T cells in the skin are expressing gzmB

In addition to cytokine production, CD8 T cells could also function as cytotoxic cells during infection. To determine if the CD8 T cells in the skin had a cytotoxic phenotype, we examined and compared the expression of gzmB, one of the important cytolytic proteins, in CD8 T cells from the DLN and *L. major* infected skin. Interestingly, CD8 T cells from the DLN were negative for gzmB expression, but the majority of CD8 T cells in the skin were expressing gzmB (Figure 2I and 2J). The expression is shown for cells incubated with BFA alone, as restimulation with PMA/Ionomycin did not significantly change the gzmB expression in either the DLN or the skin (data not shown). This data is consistent with analysis of CD8 T cells from patient lesions, where granzyme expressing CD8 T cells are a prominent cell population that is associated with increased inflammatory infiltration and disease severity (Faria et al., 2009; Novais et al., 2013; Santos et al., 2013).

***Leishmania* specific cells in the skin express gzmB but not IFN-γ**

Having examined the expression of IFN-γ and gzmB by the total CD8 T cell population in the DLN and skin, we wanted to see if the expression patterns would be similar in a *Leishmania* specific CD8 T cell. To do this, we utilized transgenic CD8 T cells that express a T cell receptor that is specific for ovalbumin, OTI T cells, and a strain of *L. major* that expresses ovalbumin (*L. major*-OVA) (Prickett et al., 2006). Various numbers of CFSE labeled OTI T cells were transferred into mice and the next day the mice were infected with *L. major* or *L. major*-OVA. After one week, OTI cells in the DLN were analyzed for dilution of CFSE as a marker for proliferation. As expected, the number of OTI T cells transferred had a large impact on the amount of division that had occurred

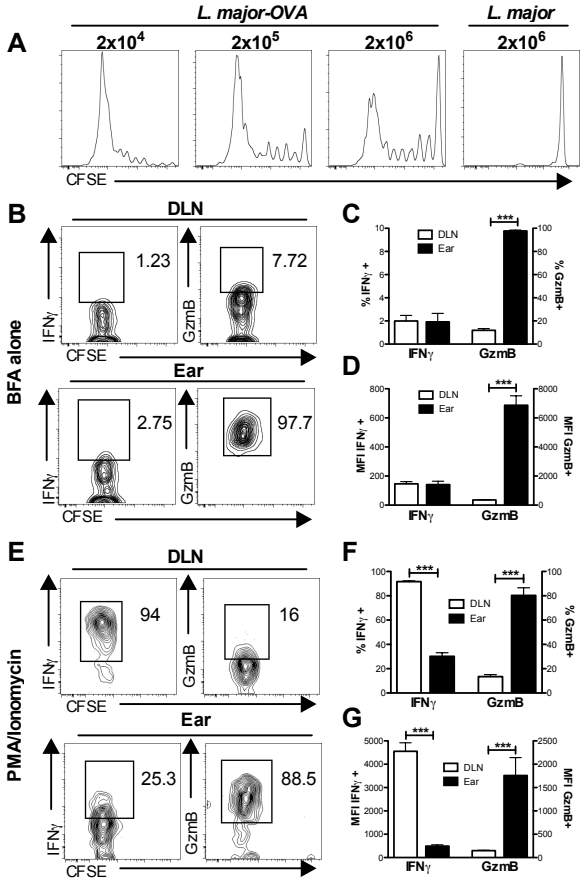

Figure 3. Antigen specific CD8 T cells in *L. major* infected skin express gzmB but not IFN-γ. Transgenic OTI CD8 T cells were isolated from the spleen and labeled with CFSE. Various numbers of OTI cells were transferred into B6 mice, which were infected the following day with *L. major* or *L. major-OVA.* After 1 week, the DLN were harvested and the OTI cells were analyzed for CFSE dilution (A). Cells from the DLN and infected skin were also incubated with BFA alone (B-D) or restimulated with PMA/ Ionomycin (E-G) and analyzed for IFN-γ and gzmB expression by flow cytometry. The percent (C and F) and MFI (D and G) of IFN-γ or gzmB producing cells in the skin and DLN were calculated. Data are representative of 3 independent experiments (A-D; n=3-4 mice per group) or 2 independent experiments (E-G; n=5 mice per group). Error bars represent the SEM.

during the first week (Badovinac et al., 2007; Foulds and Shen, 2006). When a small number of OTI cells ($2x10^4$) were transferred >95% of the cells had divided at least 7 times and diluted out their CFSE (Figure 3A). OTI T cells transferred into mice that were infected with WT *L. major* did not divide (Figure 3A). Analysis of fully divided ($CFSE^{dim}$) CD8 T cells in the DLN without restimulation showed very little expression of IFN-γ or

gzmB (Figure 3B-3D). However, upon restimulation with PMA/Ionomycin nearly all of these OTI cells in the DLN made IFN-γ (Figure 3E and 3F). As before, restimulation did not change the gzmB expression. Interestingly, OTI cells from the skin were all expressing gzmB and even after restimulation with PMA/Ionomycin did not make much IFN-γ (Figure 3B-3E). It is especially striking to notice that OTI cells in the DLN were not expressing gzmB, whereas OTI cells that had similarly diluted their CFSE in the skin were almost all gzmB+. In contrast, while OTI cells in the DLN could make IFN-γ upon stimulation, significantly fewer of the OTI cells in the skin produced IFN-γ. Given these results, we hypothesized that the environment within the leishmanial lesion was altering the phenotype and function of CD8 T cells such that they became poor cytokine producers and instead adopted a cytotoxic phenotype.

Non-specific CD8 T cells enter the lesion and express gzmB

CD8 T cells can be recruited to tissues from the blood independent of antigen specificity, which allows us to test whether the phenotype of CD8 T cells within the leishmanial lesion was dependent on antigen (Ely et al., 2003). However, it has been shown that the ability to enter uninflamed tissue occurs during a small window of time early after the initial activation of a CD8 T cell; therefore, we began by testing the ability of non-*Leishmania* specific cells of different activation states to traffic to the leishmanial lesion (Masopust et al., 2004). To ensure that the cells were not specific for *Leishmania*, we performed a transfer experiment using transgenic P14 CD8 T cells. These cells express a TCR specific for the LCMV peptide GP33. P14 T cells were transferred into CD45 congenic C57BL/6 (B6) mice, which were then infected with lymphocytic choriomeningitis virus (LCMV). The P14 T cells were harvested during the effector phase (day 5 post-infection) or memory phase (day 30 post-infection) of LCMV infection.

Naive, effector, or memory P14 T cells were then transferred into three different groups of congenic mice that had been infected with *L. major* for 2 weeks (Figure 4A). The spleen, infected skin, and uninfected control skin were analyzed for the presence of the donor cells 36 hours later. P14 T cells were readily detected in the spleen at similar frequencies in each group (Figure 4B and 4E). As predicted based on previous literature (Butcher and Picker, 1996; Masopust et al., 2004), naïve P14 T cells were unable to migrate into *L. major* infected skin (Figure 4C). Similarly, regardless of the activation state of the transferred cells, none were detected in the uninfected control skin (Figure 4D and 4E). However, both effector and memory P14 T cells were found in the *L. major* infected skin (Figure 4C and 4E).

Given that both the effector and memory P14 T cells were able to migrate into the inflamed skin, we wanted to evaluate their expression of IFN-γ and gzmB. Cells were harvested and incubated with BFA without additional stimulation. As expected, a large percentage of effector P14 cells in the spleen expressed gzmB, whereas naïve and memory P14 T cells did not (Figure 4F and 4H). P14 cells from all three groups were negative for IFN-γ in the spleen (Figure 4F and 4H). In contrast, both effector and memory P14 T cells in the infected ear expressed gzmB and IFN-γ, although the IFN-γ levels were quite low (Figure 4G and 4H). These data suggest that both effector and memory T cells not only migrate into *Leishmania* lesions, but also become activated upon entering the inflamed tissue. It also provides further evidence that CD8 T cells in the skin during *Leishmania* infection, regardless of antigen specificity, preferentially express gzmB over IFN-γ.

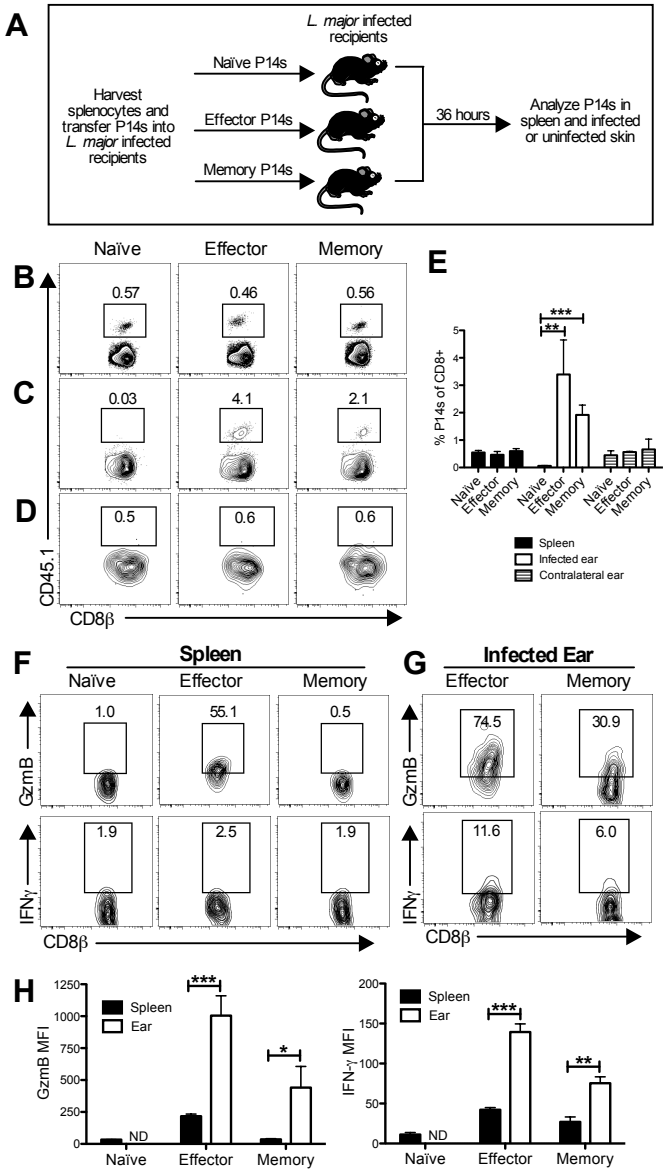

Figure 4. LCMV memory T cells migrate to leishmanial lesions and upregulate gzmB expression. B6 mice received CD45.1+ P14 cells (P14 chimeras) and were infected with LCMV for 5 days (effectors) or 30 days (memory). At the indicated time post LCMV infection, splenocytes were harvested from naïve P14 mice or P14 chimeras and the numbers of P14 CD8 T cells were quantified. Equal numbers ($5x10^5$) of P14 CD8 T cells were transferred into congenically marked B6 mice that had been infected with *L. major* for 2 weeks (A). After 36 hours, spleens (B), infected ears (C), and contralateral uninfected ears (D) were harvested from the recipients and P14 frequency was analyzed by flow cytometry (E). P14 CD8 T cells present in the spleen (F) or infected ear (G) were incubated with BFA alone for 5 hours and analyzed for production of gzmB and IFN-γ. The relative MFI for each was calculated (H). Data are representative of two independent experiments (n=3-5 mice/ group). Error bars represent the SEM.

Cytokine signals responsible for IFN-γ or gzmB expression by CD8 T cells

In addition to responding to specific antigens, effector and memory phenotype CD8 T cells can be activated by the cytokine milieu present during inflammation (Freeman et al., 2012). There is much evidence for the role of cytokines in the functional programming of CD8 T cells both in vitro and during various types of infections. One of the most potent and well-studied combinations of cytokines that induce IFN-γ production is IL-12 and IL-18 (Berg et al., 2003; Bou Ghanem et al., 2010; Freeman et al., 2012; Kambayashi et al., 2003; Raué et al., 2004; Raué et al., 2013). Less is known about the effect of the cytokine environment on gzmB expression. We wanted to examine the ability of activated CD8 T cells in *L. major* infected mice to respond to various cytokines in order to see if there was a cytokine signal that would differentially regulate IFN-γ and gzmB expression within CD8 T cells similarly to what we saw in the skin during *L. major* infection. We incubated splenocytes with cytokines for 36 hours, added BFA to the cultures for the final 6 hours of the incubation, and measured the production of IFN-γ or gzmB by staining the cells for flow cytometry (Figure 5A and 5C) or analyzing the culture supernatants (Figure 5B and 5D). As expected, the combination of IL-12 and IL-18 induced large amounts of IFN-γ production by CD8 T cells as well as a moderate amount of gzmB (Figure 5A and 5B). The combination of IL-2 and IL-18 also had a synergistic effect on gzmB production, inducing over half of the $CD44^{hi}$ CD8 T cells in the culture to express gzmB (Figure 5C). Interestingly, while IL-15 treatment did not induce any IFN-γ production, it had a potent effect on gzmB expression (Figure 5C and 5D). This is consistent with findings that IL-15 can enhance the cytolytic activity of memory CD8 T cells during Listeria infection (Soudja et al., 2012).

Having identified IL-12 + IL-18 and IL-15 as potential IFN-γ and gzmB inducers respectively, we repeated these in vitro stimulations to further interrogate the effect of

these cytokines on the IFN-γ and gzmB production by CD8 T cells from mice with different immunological histories. Despite using CD44 as a marker for activation, we predicted that $CD44^{hi}$ CD8 T cells from a naïve mouse would respond to cytokines differently than those from a mouse with an active infection or one with infection induced memory cells. To test this, we incubated splenocytes from naïve, *L. major* infected (4 weeks post infection), or lymphocytic choriomeningitis virus (LCMV) immune (4 weeks post infection with acute strain) mice with cytokines for 12 (Figure 5E) or 36 (Figure 5F) hours and examined the expression of IFN-γ or gzmB by $CD44^{hi}$ CD8 T cells in each group. After only 12 hours, we again observed that IL-12 + IL-18 induced IFN-γ but very little gzmB expression while IL-15 had the exact opposite effect (Figure 5E). CD8 memory T cells from LCMV immune mice were better able to respond to IL-12 + IL-18 at this early time point than cells from naïve or *L. major* infected mice, as evidenced by increased expression of IFN-γ (Figure 5E). This is consistent with the observation that cytokine gene expression remains high in memory cells, allowing for rapid production of cytokines upon reactivation (DiSpirito and Shen, 2010; Kaech et al., 2002). At 36 hours, as we saw before, IL-12 + IL-18 induced high amounts of IFN-γ and also induced some expression of gzmB (Figure 5F). Strikingly, while prolonged exposure to IL-15 dramatically increased the amount of gzmB expression by CD8 T cells in all 3 groups of mice, none of these cells expressed IFN-γ (Figure 5F). Taken together these data provide evidence that differently activated $CD44^{hi}$ CD8 T cells can respond to cytokine signals uniquely and that IL-15 is a potential candidate for a signal that induces gzmB expression but not IFN-γ in the skin during *L. major* infection.

IL-15 is expressed in the skin

IL-15 is a unique cytokine in that it is thought to be trans-presented on the surface of

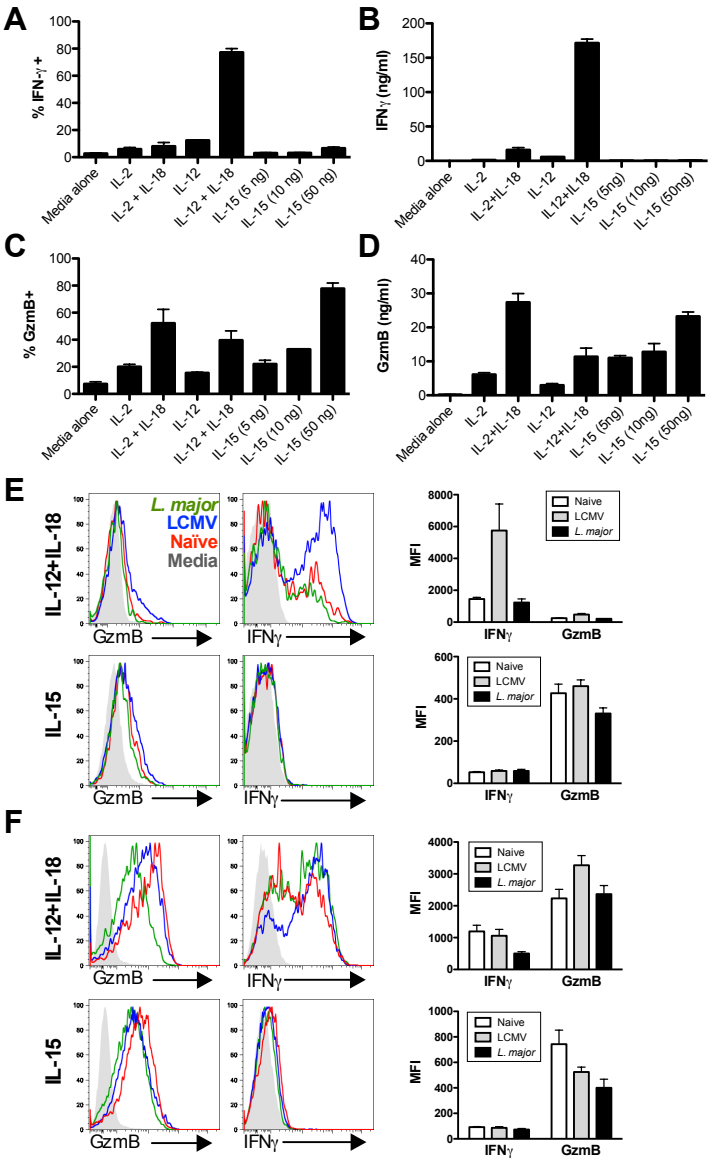

Figure 5. IL-15 induces gzmB expression but not IFN-γ. B6 mice were infected with *L. major* and 4 weeks after infection, splenocytes were harvested, and cells were cultured with various cytokines or media alone for 36 hours. BFA was added for the final 6 hours of culture. Supernatants were removed for analysis by ELISA (B and D) and cells were analyzed for IFN-γ and gzmB expression by flow cytometry (A and C). B6 mice were infected with LCMV or *L. major* for 4 weeks or left uninfected, splenocytes were harvested, and cells were cultured with IL-12 + IL-18 or IL-15 for 12 hours (E) or 36 hours (F). BFA was added for the final 6 hours of culture. Cells were gated on $CD44^{hi}$ CD8+ T cells and analyzed for IFN-γ and gzmB expression by flow cytometry and MFI for each was calculated (E and F). Data are representative of 2 independent experiments (A-D; n=3 mice/group) or 3 independent experiments (E-F; n=5 mice/group). Error bars represent the SEM.

cells by binding the high affinity subunit of its receptor, IL-15Rα (Stonier and Schluns, 2010). This unique feature allows the surface expression of IL-15Rα to be used as a marker for cells that could trans-present IL-15. Using targeted expression of IL-15Rα, it has been shown that dendritic cells (DCs) are an important source of IL-15 trans-presentation to CD8 T cells (Stonier and Schluns, 2010). We therefore examined DCs in the skin for expression of IL-15Rα to determine if IL-15 could be playing a role during infection. DCs from both naïve and *L. major* infected skin express fairly high levels of IL-15Rα (Figure 6A). The expression levels on DCs in *L. major* infected skin are lower as assessed by MFI, however it is important to remember that the numbers of DCs in infected skin compared to naïve skin are much higher, resulting in an overall increase in the number of IL-15Rα expressing cells following infection.

IL-15 is required for the early expression of gzmB

To test whether IL-15 is playing a role in the gzmB expression by CD8 T cells in the skin during *L. major* infection, we once again used a transfer system. To minimize the impact of TCR driven expression of gzmB, we transferred CD8 T cells from LCMV immune mice into mice that were infected with *L. major* (Figure 6B). While these cells were not transgenic, the majority of activated cells in these mice are LCMV specific and LCMV has been shown to have no detectable cross reactivity with *L. major* (Chapter 3). To test the role of IL-15 on expression of gzmB in the skin, cells were transferred into WT or IL-15KO mice. Prior to transfer, resting LCMV memory cells were not expressing gzmB (Figure 6C). After 36 hours, a population of transferred cells was detectable in the *L. major* infected skin. Over half of the LCMV memory CD8 T cells from the WT mouse were expressing gzmB, while significantly fewer of the cells transferred into the IL-15KO mice were (Figure 6D). This decrease was seen not only in the percent of cells

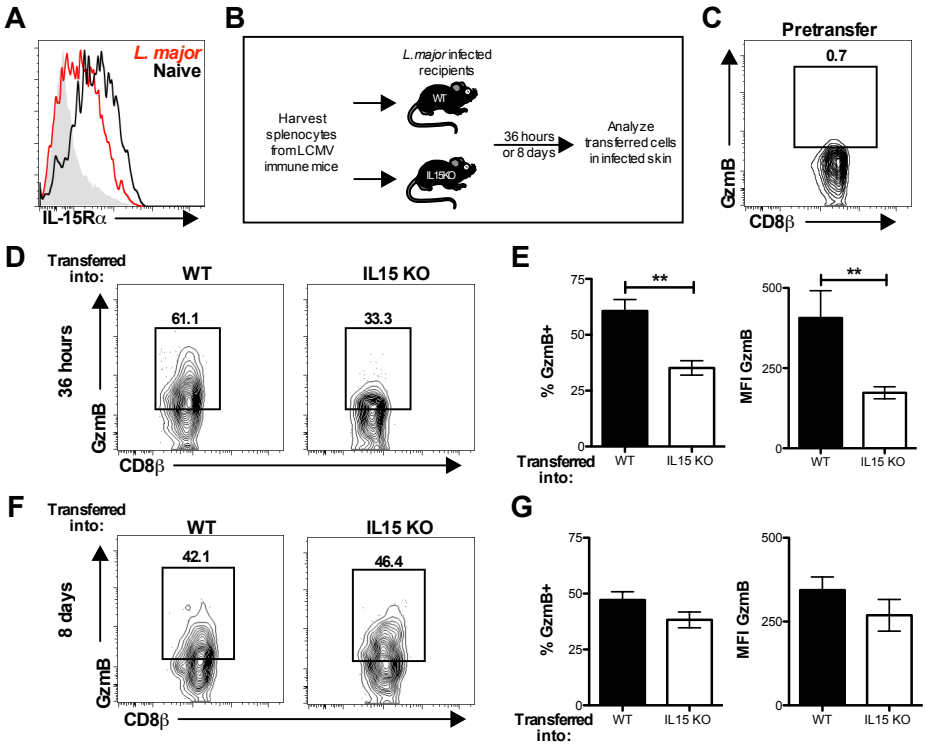

Figure 6. IL-15 is required for the early induction of gzmB in *L. major* infected skin. B6 mice were infected with *L. major* and 1 week post infection the skin was harvested and analyzed by flow cytometry (A). Plot was pregated on live, CD45+, CD11c+ cells. B6 mice were infected with LCMV and 30 days post infection splenocytes from LCMV immune mice were harvested and transferred into WT or IL-15KO *L. major* infected mice (B). GzmB expression was assessed on cells prior to transfer (C). After 36 hours (D-E) or 8 days (F-G), infected ears were harvested, incubated with BFA, and gzmB expression by transferred cells was analyzed by flow cytometry. The percent of gzmB+ cells and MFI of gzmB expression were calculated (E and G). Data are representative of 1 experiment (n=5 mice/group). Error bars represent the SEM.

expressing gzmB, but also in the MFI of gzmB expression (Figure 6E). However, if the cells were examined 8 days post transfer, the expression of gzmB was the same whether IL-15 was present in the recipient or not (Figure 6F and 6G). This suggests that IL-15 is playing a role in the early activation of CD8 T cells in the skin to express gzmB, but that over time other signals are able to compensate such that, even in the absence of IL-15, gzmB expression is induced.

2.5 Discussion

CD8 T cells have been demonstrated to play an important role producing IFN-γ in the DLN following *L. major* infection, but little was known about their function in the skin (Uzonna et al., 2004). As a barrier surface, the skin is an important immunological niche and is the site of many other types of infections and autoimmune disorders (Gebhardt et al., 2011; Gudjonsson et al., 2004; Harris et al., 2012; Hijnen et al., 2013; Mackay et al., 2012). Not surprisingly, analysis of normal human skin revealed that there are ~$1x10^6$ T cells per cm^2 of skin, presumably acting as a first line of defense against pathogens (Clark et al., 2006). We can also detect a significant population of both CD4 and CD8 T cells in normal human skin samples (unpublished data). Despite this, in naïve mice CD8 T cells are virtually absent from uninflamed skin (Figure 1A). This discrepancy between human and mouse skin has important implications as we use this model to study immune responses in the skin.

Here we have shown that CD8 T cells are recruited to the skin very early following infection with *L. major.* Contrary to our predictions, these CD8 T cells do not appear to be making much IFN-γ in the skin, but rather are expressing the cytotoxic protein gzmB. This phenotype was true of total CD8 T cells present in lesions, antigen specific transgenic CD8 T cells, and non-specific bystander CD8 T cells that were recruited to the site of inflammation (Figures 2-4). The production of gzmB rather than IFN-γ by CD8 T cells was especially surprising given that killing of infected cells by CD8 T cells has been shown to not impact the viability of parasites within those cells, suggesting that CD8 mediated killing provides no protective advantage for the host (Smith et al., 1991). Recent work from our lab demonstrated that disease progression and metastasis during *Leishmania braziliensis* infection was independent of parasite burden, but rather was

dependent on CD8 T cells (Novais et al., 2013). Expression of IFN-γ by these cells played no role in disease severity, however perforin expression was required. Interestingly, this data is also consistent with analysis of cutaneous lesions from patients. Granzyme expressing CD8 T cells could be readily found in lesion biopsies and increased numbers of these cells were correlated with more severe disease (Dantas et al., 2013; Faria et al., 2009). Together these data demonstrate that CD8 T cells within *Leishmania* infected skin adopt a cytotoxic phenotype that promotes immunopathology and does not control parasite growth.

Our in vitro studies have also examined the responsiveness of differently primed cells to cytokine stimulation. Despite the use of CD44 broadly as a marker of activation, all cells that express CD44 are not equal. Use of panel of activation markers and cytokine receptors would be needed to truly appreciate the heterogeneity of this population. While we have not exhaustively compared the CD44 expressing populations of CD8 T cells from a naïve, *L. major* infected, or LCMV immune mice, we have shown that they respond with different kinetics to cytokine stimulation (Figure 5). It is interesting to consider the implications this observation has on the heterogeneity of immune responses seen in patients, even those infected with the same pathogen. Our prediction given this data is that the repertoire of naïve, effector, and memory T cells present in each individual, not just of different specificities but also of different responsiveness to various inflammatory cytokines, has the potential to significantly impact the overall magnitude, speed, and effectiveness of their immune response.

Given the detrimental role CD8 T cells appear to be playing in the skin during *Leishmania* infection, we wanted to examine the inflammatory signals that might be responsible for inducing this cytolytic and ultimately pathologic phenotype. Classically,

CD8 T cells are thought to be both cytotoxic and cytokine producers rather than exhibiting one function or the other; however, recently high throughput single-cell analysis of CD8 T cells revealed that most CD8 T cells are actually discordant for these functions (Varadarajan et al., 2011). Those cells that secreted IFN-γ were not cytotoxic and vice versa, supporting our data that CD8 T cells in the skin can be cytolytic but not producing IFN-γ. There is also a tremendous amount of data that demonstrates the impact that the cytokine milieu has on both the priming of immune responses and the reactivation of CD8 T cells during subsequent, even unrelated infections (Freeman et al., 2012; Raué et al., 2013). Our examination of cytokines here revealed IL-15 as a potential inflammatory signal that could induce gzmB expression without IFN-γ. Transfer of non-specific CD8 T cells into IL-15KO mice infected with *L. major* confirmed a role for IL-15 in the early expression of gzmB but at later time points it was not required for the expression of gzmB by CD8 T cells in the skin. This data identifies IL-15 as a potential target for therapeutic manipulation, but also raises additional questions about what other inflammatory signals might be responsible for the pathologic programming of CD8 T cells during *Leishmania* infection. In total, CD8 T cells seem to play a protective role as cytokine producers in the DLN, but simultaneously act as pathologic cytotoxic cells in the skin. A better understanding of this dichotomy will be necessary for improved, targeted therapeutic options and safe, effective vaccine strategies.

Chapter 3 Engagement of NKG2D on Bystander Memory CD8 T Cells Promotes Increased Immunopathology Following *Leishmania major* Infection

3.1 Abstract

One of the hallmarks of adaptive immunity is the development of a long-term pathogen specific memory response. While persistent memory T cells certainly impact the immune response during a secondary challenge, their role in unrelated infections is less clear. To address this issue, we utilized lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* immune mice to investigate whether bystander memory T cells influence *Leishmania major* infection. Despite similar parasite burdens, LCMV and *Listeria* immune mice exhibited a significant increase in leishmanial lesion size compared to mice infected with *L. major* alone. This increased lesion size was due to a severe inflammatory response, consisting not only of monocytes and neutrophils, but also significantly more CD8 T cells. Many of the CD8 T cells were LCMV specific and expressed gzmB and NKG2D, but unexpectedly expressed very little IFN-γ. Moreover, if CD8 T cells were depleted in LCMV immune mice prior to challenge with *L. major,* the increase in lesion size was lost. Strikingly, treating with NKG2D blocking antibodies abrogated the increased immunopathology observed in LCMV immune mice, showing that NKG2D engagement on LCMV specific memory CD8 T cells was required for the observed phenotype. These results indicate that bystander memory CD8 T cells can participate in an unrelated immune response and induce immunopathology through an NKG2D dependent mechanism without providing increased protection.

3.2 Introduction

Over time and with increased immunological experience, our pool of memory CD8 T

cells increases, resulting in a large repertoire of memory T cells that are able to protect against previously encountered infectious agents. This protection is thought to be life long and pathogen specific. Less well studied is the ability of these memory T cells to respond in a TCR-independent fashion that might influence the outcome of an unrelated infection. A role for bystander memory T cells (i.e. memory T cells that are activated independent of TCR stimulation) has been described in viral infections, where subsequent heterologous viral challenge leads to reactivation of memory CD8 T cells and increased protection (Kohlmeier et al., 2010). Similarly, activation of bystander memory CD8 T cells has also been observed in bacterial and parasitic infections, leading to the notion that an accumulation of memory CD8 T cells may promote increased resistance to unrelated infections (Berg et al., 2003; Chu et al., 2013; Polley et al., 2005; Soudja et al., 2012). Work from several groups has shown that CD8 T cells have a remarkable ability to become activated by cytokines in a TCR-independent manner, characterized by rapid acquisition of effector functions (Freeman et al., 2012; Marshall et al., 2010; Raué et al., 2004). However, while memory CD8 T cells can promote increased resistance, in some situations activation of bystander CD8 T cells may be pathologic and has even been shown to play a role in autoimmune diseases (Meresse et al., 2004). The inflammatory signals that induce a bystander CD8 T cell to be protective versus pathologic in different disease states is poorly understood.

Cutaneous leishmaniasis has a wide spectrum of clinical presentations, from mild self-healing lesions to severe chronic infections. Control of these parasites is primarily dependent upon the development of a strong CD4 Th1 response, which leads to the production of IFN-γ that activates macrophages and kills the parasites (Kaye and Scott, 2011; Sacks and Noben-Trauth, 2002). Under some conditions, CD8 T cells also play a protective role by producing IFN-γ to both directly activate macrophages, and promote

the development of a strong CD4 Th1 response (Belkaid et al., 2002; Uzonna et al., 2004). However, disease severity in leishmaniasis is only partially dependent upon the parasite burden, and some forms of the disease are associated with very few parasites but an exaggerated immune response (Lessa et al., 2007; Murray et al., 2005; Pearson and Sousa, 1996). The factors that determine the severity of the disease remain poorly defined, but may include decreased expression of IL-10 or the IL-10R, thereby leading to increased production of IFN-γ, TNF-α and/or IL-17 (Bacellar et al., 2009; Boaventura et al., 2010; Gonzalez-Lombana et al., 2013; Oliveira et al., 2012; Oliveira et al., 2011). Additionally, in some patients there is a strong correlation between the severity of the disease and the number of CD8 T cells within the lesions (Brodskyn et al., 1997; Faria et al., 2009; Santos et al., 2013). Instead of expressing IFN-γ, however, the majority of these CD8 T cells express gzmB (Faria et al., 2009; Santos et al., 2013). Recently, we have shown that these cytolytic CD8 T cells promote pathology, rather than resistance (Novais et al., 2013). Thus, while IFN-γ producing CD8 T cells may be protective in leishmaniasis, it appears that gzmB expressing CD8 T cells are associated with enhanced disease.

In this study, we found that bystander CD8 memory T cells exacerbate disease following infection with *L. major*. We infected mice with LCMV or *Listeria* to generate a large pool of memory CD8 T cells, and challenged the mice with *L. major*. Following infection with *L. major*, LCMV or *Listeria* immune mice develop significantly larger lesions than control mice characterized by increased numbers of monocytes, neutrophils, and CD8 T cells but no change in the parasite burden. Depletion of CD8 T cells following LCMV infection, but prior to challenge with *L. major*, resulted in a loss of the observed pathology. Strikingly, when anti-NKG2D blocking antibodies were given, the increased immunopathology in LCMV immune mice was completely abrogated, indicating that

engagement of the NKG2D pathway in CD8 T cells was responsible for the observed immunopathology. Thus, this work demonstrates that a bystander memory CD8 T cell population can contribute to the chronic inflammation and immunopathology associated with cutaneous leishmaniasis.

3.3 Materials and methods

Animals

Female C57BL/6 mice and B6-Ly5.2/Cr (CD45.1) (6 weeks old) were purchased from the National Cancer Institute (Fredericksburg, MD). RAG2/OT-I mice were purchased from Taconic Farms. Mice expressing eGFP in all T cells were originally obtained from Ulrich van Andrian (Harvard University) and crossed with RAG2/OTI mice to generate GFP+ OTI CD8 T cells. CD45.1+ P14 mice bearing the H-2D^{b} gp33-specific T cell receptor were maintained in our animal colony (Pircher et al., 1989). Animals were housed in a specific pathogen-free environment and tested negative for pathogens in routine screening. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01.

Parasite, virus, and bacterial infections

L. major parasites (Friedlin) or *L. major* expressing ovalbumin (*L. major*-OVA) were grown to the stationary phase in Schneider's Drosophila medium (Gibco) supplemented with 20% heat-inactivated FBS (Gibco) and 2 mM L-glutamine (Sigma) at 26°C (Prickett et al., 2006). Metacyclic promastigotes were isolated from 4-5 day old stationary cultures

by density gradients (Späth and Beverley, 2001). Mice were infected with 2 x 10^6 metacyclic parasites injected intradermally into the ear. Lesion development was monitored weekly by taking measurements of ear thickness with digital calipers (Fisher Scientific). Parasite burden in lesion tissues was assessed using a limiting dilution assay as previously described (Scott et al., 2004). For viral infections, mice were infected with 2 x 10^5 PFU of LCMV Armstrong strain by i.p. injection. For bacterial infections, mice were prime-boosted with an initial infection of 10^3 *Listeria monocytogenes* expressing ovalbumin (*Listeria*-OVA) followed 30 days later by an additional boost infection of 5 x 10^4 *Listeria*-OVA, both doses given intravenously.

T cell transfers

P14 chimeras were made by transferring 2 x 10^4 congenically marked P14 CD8 T cells 1 day prior to infection with LCMV. Prior to transfer of effector or memory P14 CD8 T cells from P14 chimeras, splenocytes were enriched for T cells using the Pan T cell Isolation Kit II according to the manufacturer's instructions (Miltenyi Biotec). Enriched fractions were analyzed by flow cytometry and 5 x 10^5 P14 CD8 T cells were transferred. Naïve P14 CD8 T cells were not enriched prior to transfer. When OTI CD8 T cells were used, 2 x 10^5 GFP+ cells were transferred 1 day prior to *L. major*-OVA infection.

Histology

L. major infected ears were taken at the peak of lesion formation, fixed in 10% buffered formalin, and embedded in paraffin. Longitudinal 5 μm sections were cut and stained with hematoxylin and eosin or Verhoeff's stain. For analysis of Rae1-γ, skin was taken at the peak of lesion formation and frozen in Tissue-Tek OCT compound (Electron Microscopy Sciences). Longitudinal 5 μm sections were cut and stained with biotinylated anti-mouse Rae1-γ (eBioscience, CX1). Sections were blocked prior to

staining with Avidin Biotin blocking solution according to the manufacturer's instructions (Thermo Scientific). Photographs were taken with a Nikon Digital Sight DS-Fi1 Color system, (Nikon eclipse E600 Microscope).

Flow cytometry

For flow cytometry, cells were isolated from ears, draining lymph nodes, spleens or peripheral blood. For ears, dermal sheets were separated and incubated in incomplete IMDM+GlutaMAX (Gibco) containing 0.25 μg/mL of Liberase TL (Roche, Diagnostics Corp.) and 10 μg/mL DNase I (Sigma-Aldrich) for 90 minutes at 37°C. Ears, draining lymph nodes, and spleens were mechanically dissociated by smashing through a 40-μm cell strainer (Falcon) in PBS containing 0.05% BSA and 20 μM EDTA. Splenocytes were incubated for <1 minute with ACK lysing buffer (Lonza) to lyse red blood cells. When indicated, cells were incubated at 4 x 10^6 cells/ml with Brefeldin A (BFA, 3 μg/ml final concentration) (eBioscience) alone for 5 hours, with a pool of 20 LCMV peptides (each peptide at a final concentration of 0.4 μg/ml) and BFA for 6 hours, or with phorbol myristate acetate (PMA) (Sigma, 100 ng/ml final concentration) and ionomycin (Sigma, 1 μg/ml final concentration) and BFA for 4-6 hours before staining for flow cytometry. In experiments analyzing CD107a expression, cells were incubated with BFA, monensin (eBioscience, 2 μM final concentration), and anti-CD107a (eBioscience) for 6 hours. Fixable Aqua dye (Invitrogen) was added to assess cell viability. Cells were then incubated with FC block (anti-CD16/32, heat inactivated mouse sera and Rat IgG) followed by fluorochrome-conjugated antibodies for surface markers CD45, CD45.1, CD45.2, CD8β, CD4, CD44, CD62L, CD11b, Ly6C, and/or Ly6G (1A8) (all eBioscience) and were fixed with 2% paraformaldehyde (Electron Microscopy Sciences). For intracellular staining, cells were previously permeabilized with 0.2% of saponin buffer and stained for IFN-γ, gzmB, TNF-α, and/or IL-17A (eBioscience or Invitrogen). The data

were collected using an LSR Fortessa flow cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star). For sorting LCMV memory cells, splenocytes were stained for surface markers (CD4, CD8β and CD44) and $CD8\beta^{+}$ $CD44^{hi}$ cells were sorted on a FACSAria II (BD Biosciences).

Histopaque

Peripheral blood was collected into a 4% sodium citrate solution. White blood cells were isolated by underlaying with Histopaque-1083 (Sigma) and spinning for 20 minutes at 400 x g at room temperature.

Ear Homogenization

Whole ears were cut into <1 mm pieces and placed in ice cold PBS with a protease inhibitor cocktail (Sigma). Samples were homogenized using the FastPrep-24 (MP Biomedicals) and spun for 20 minutes at 13000 rpm at 4°C in a microcentrifuge. The supernatants were removed and stored at -80°C until analysis by ELISA as described below.

Leishmanial antigen restimulation and ELISAs

Leishmanial antigen was obtained from stationary-phase promastigotes of L. major by resuspending parasites at 1 x 10^9 parasites/ml in PBS and conducting 20 freeze/thaw cycles. For measurements of antigen-specific cytokine production, the infected skin draining retroauricular lymph node was removed, mechanically dissociated, and single cell suspensions were prepared. Cells were resuspended in complete IMDM+GlutaMAX (Gibco) supplemented with 10% heat inactivated FBS (Gibco), 2 mM L-glutamine (Sigma), 100 U of penicillin and 100 μg of streptomycin (Sigma) per mL and 0.05 μM of β-mercaptoethanol (Sigma). Cells were plated at 4 x 10^6 cells/mL in 1 ml in 48-well

plates. Cells were incubated at 37°C in 5% CO_2 with 20 x 10^6 L. major parasites/mL. Supernatants were collected after 72 hours and stored at -20°C until they were assayed by sandwich ELISA using paired monoclonal antibody to detect IFN-γ, IL-4 or IL-17 (eBioscience). Cytokine concentrations were calculated from standard curves with detection limit of 0.03 ng/mL for IFN-γ, 0.015 ng/mL for IL-17A and 7 Units/mL of IL-4. Granzyme B was analyzed by ELISA using a mouse granzyme B Duoset kit (R&D Systems).

VITAL assay

A VITAL assay was performed and analyzed as described (Hermans et al., 2004). Briefly, CD45.1+ CD8 T cells for the VITAL assay were enriched from the lesions of LCMV immune L. major infected mice at 4 weeks post L. major infection using negative selection kit for CD8 T cells according to the manufacturer's instructions (Miltenyi Biotec). Target cells for this assay were control RMA cells that do not express any NKG2D ligands or RMA cells that had been stably transfected with Rae1, originally made by L. Lanier (Cerwenka et al., 2001). RMA control cells were labeled with a high dose (1 μM) of CTV (Cell trace violet) and Rae1 expressing RMA cells were labeled with a low dose (0.02 μM) of CTV for use as target cells according to the manufacturer's instructions (Life Technologies). Control and Rae1 expressing cells were mixed 1:1 and incubated with LCMV immune CD8 T cells from the lesion at an effector to target ratio of 4:1 for 5 hours in vitro. The CTV low-to-high ratio of live cells was determined by analysis on an LSR Fortessa flow cytometer. Dead cells were excluded by propidium iodide staining. Expression of Rae1 was confirmed using a pan-Rae1 antibody (R&D systems).

In vivo antibody treatment

For CD8 depletion, C57BL/6 mice were either infected with LCMV or left uninfected for 45 days. Both groups were then treated with 250 µg anti-CD8 (Clone 53-6.72; BioXCell) in PBS every 3 days for 15 days for a total of 5 doses. On the final day of antibody treatment, all groups were infected with *L. major* as described above. NK1.1 depleting (500 µg/dose; Clone PK136; BioXCell), NKG2D blocking (200 µg/dose; Clone HMG2D; BioXCell), and Hamster IgG control (200 µg/dose; BioXCell) antibodies were given 1 day prior to infection with *L. major* and twice weekly for the duration of the experiment.

Statistics

Results represent means ± SEM. Data were analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined using unpaired, one-tailed Student's *t* test with p values given as: *$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$; ns $p > 0.05$. Results with a p value ≤0.05 were considered significant.

3.4 Results

Previous heterologous infection exacerbates the immunopathology of *L. major* infection

Having shown previously that LCMV memory T cells readily entered the skin in response to *Leishmania* infection (Chapter 2, Figure 4), we wanted to determine if previous infection with LCMV affected the course of subsequent *L. major* infection. For this, we infected B6 mice with LCMV or left them uninfected and waited thirty days for the infection to clear and a stable memory T cell population to form. All mice were then infected with *L. major* and lesion development was monitored over time. Given that LCMV induces a strong Th1-type immune response, we predicted that the LCMV immune mice would be more resistant to *L. major* infection. However, LCMV immune

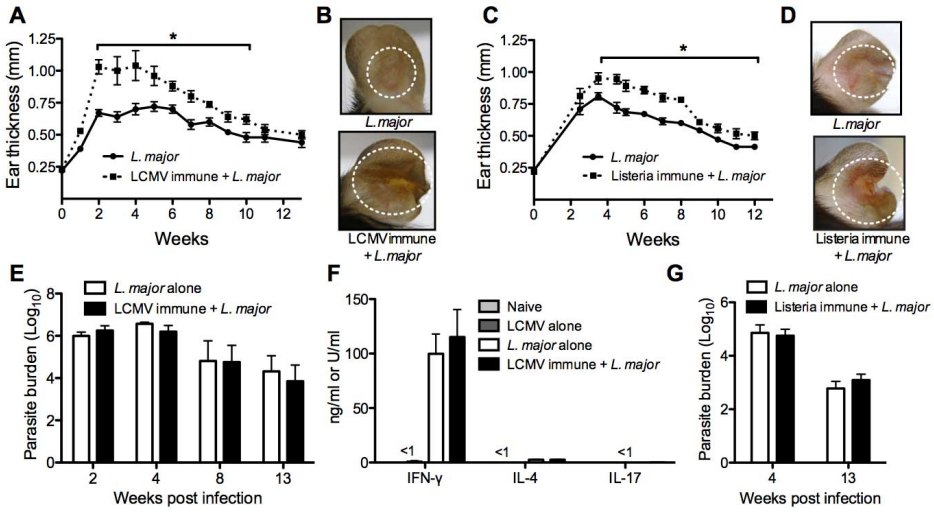

Figure 1. Previous heterologous infection increases leishmanial lesion size with no effect on parasite control. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, mice were infected with *L. major* and ear thickness was measured weekly (A). Pictures were taken 4 weeks post *L. major* infection (B). B6 mice were infected with *Listeria*-OVA or left uninfected and 30 days later boosted again with *Listeria*-OVA or left uninfected. Thirty days after the boost, all mice were infected with *L. major* and ear thickness was measure weekly (C). Pictures were taken 5 weeks post *L. major* infection (D). Infected skin from both groups was taken at various time points post infection and parasite burden was assessed using a limiting dilution assay (E and G). At 4 weeks post infection in the LCMV immune mice, draining lymph nodes were removed and cultured with leishmanial antigen or media alone. After 72 hours, supernatants were removed and analyzed for IFN-γ, IL-4, or IL-17 by ELISA (F). Cells cultured with media alone did not produce any cytokines (data not shown). These data are a compilation of five independent experiments (n=5-10 mice per group per time point; A, B, E, and F) or representative of two independent experiments (n=10-16 mice; C, D, and G).

mice challenged with *L. major* had significantly larger lesions than did mice that had received *L. major* alone, and the lesions exhibited exacerbated gross pathology (Figure 1A and 1B). In some mice the severe inflammatory response led to permanent loss of tissue. Despite this increase in lesion size, there were no differences in parasite burden within the lesions between the two groups at any time point examined (Figure 1E). In summary, not only were the LCMV immune mice susceptible to infection, the lesions were larger and more severe than mice that received *L. major* alone.

To determine if this was a phenotype specific to LCMV, we did similar experiments with *Listeria monocytogenes*. *Listeria* immune or naïve mice were infected with *L. major* and lesion development was monitored. As with the LCMV immune mice, *Listeria* immune mice had larger lesions with greater pathology than did control mice, with no corresponding change in parasite burden (Figure 1C, 1F, and 1G). Taken together, these unexpected data show that previous heterologous infections can significantly exacerbate the disease associated with *L. major* infection, without any increased ability to control the parasite.

Leishmania-specific cytokine responses are unaffected by previous infection with LCMV

To determine if a previous LCMV infection impacted the systemic anti-leishmanial response, cells from the draining lymph node were isolated at various times after *L. major* infection from control and LCMV immune mice, and cultured with either media alone or leishmanial antigen. Supernatants were collected at 72 hours and analyzed for the presence of IFN-γ, IL-4 or IL-17 by ELISA (Figure 1F). As expected, cells from *L. major* infected mice produced significant levels of IFN-γ in response to leishmanial antigen. Similar levels of IFN-γ were produced in response to stimulation by cells from LCMV immune *L. major* infected mice, which is consistent with the fact that there were no differences in parasite control between mice that had previously been infected with LCMV and controls. Correspondingly, the levels of IL-4 and IL-17 were quite low in both groups of mice. When lymph node cells from control naïve or LCMV immune mice were cultured with leishmanial antigen, very low levels of IFN-γ and no IL-4 or IL-17 were detected (Figure 1F). Data shown here is from 4 weeks post *L. major* infection, however the same results were also seen at earlier (1-2 weeks) and later (8-12 weeks) time points throughout the infection (data not shown).

Increased infiltration into the lesions of LCMV immune *L. major* infected mice

To understand the nature of the increased pathology seen in LCMV immune mice infected with *L. major*, we examined the cellular infiltration into the lesions. Staining leishmanial lesions in control and LCMV immune mice with hematoxylin and eosin identified an infiltration of leukocytes, predominantly of mononuclear and polymorphonuclear cells. However, the magnitude of the inflammatory response was significantly greater in the lesions from LCMV immune mice (Figure 2A). In addition, to get a better understanding of the structural changes occurring in the lesions from LCMV immune mice, a Verhoeff's stain for extracellular matrix proteins was used. These sections exhibited epidermal thickening (hyperplasia) and spongiosis (Figure 2B), decreased dermal collagen and increased ulceration (Figure 2C and 2D), as well as destruction of the ear cartilage (Figure 2C). To further characterize the cellular infiltrate, we analyzed the cellular composition of the cells in the lesions by flow cytometry. As expected from the histology, we saw a significant increase in the number of both monocytes and neutrophils present in the lesions of LCMV immune *L. major* infected mice compared to controls (Figure 2E and 2F).

Further analysis by flow cytometry revealed that there was also a significant increase in the number of CD8 T cells in the lesions of LCMV immune mice (Figure 2G and 2H). The increase in CD8 T cells was seen as early at 2 weeks post *L. major* infection and was maintained throughout the entire course of infection (data not shown). In contrast, there was no change in the number of CD4 T cells present in the lesion (Figure 2G and 2H). Similar to CD4 T cells, the number of NK and NKT cells was not different in the two groups (Figure 2I and 2J).

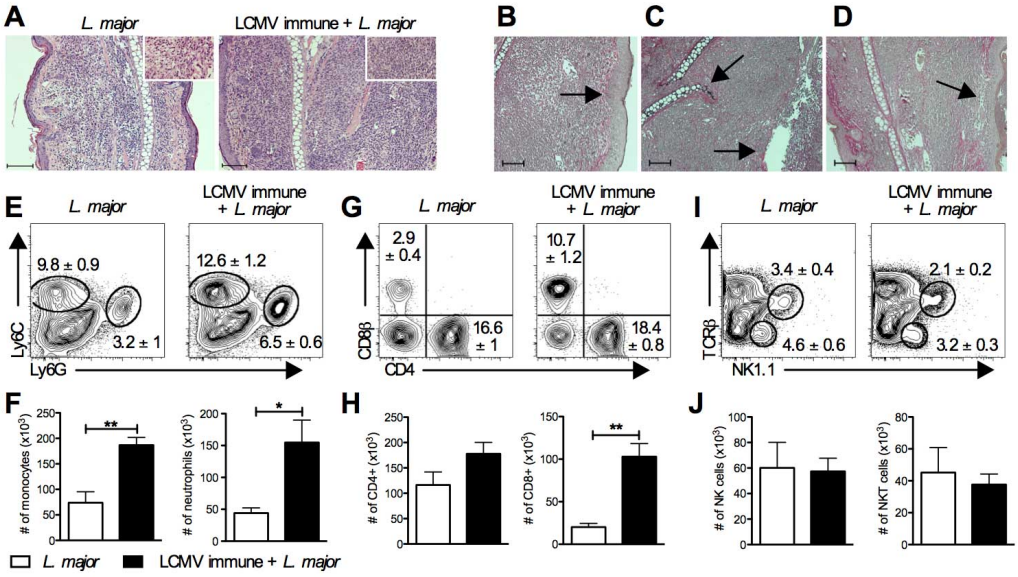

Figure 2. Increased cellular infiltration into the lesion of LCMV immune *L. major* infected mice. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, mice were infected with *L. major.* After 4 weeks, infected ears were taken and fixed for histological analysis (A-D) or digested for flow cytometric analysis (E-H). Fixed ear tissue was sectioned and stained with hematoxylin and eosin (H&E) and imaged at 10x magnification or 40x magnification (inlay)(A). A Verhoeff's stain was also done on fixed ear tissue from LCMV immune *L. major* infected mice to stain for collagen and imaged at 10x magnification (B-D). Scale bars represent 100 μm. Damage unique to the LCMV immune lesions is highlighted by arrows, specifically epidermal thickening (B), cartilage destruction (C), epidermal ulceration (C), and loss of dermal collagen (D). Digested ears were stained for surface markers of monocytes and neutrophils (E), T cells (G), or NK cells (I). Results are shown in the form of representative plots (E, G, and I) and total cell numbers (F, H, and J). Samples were pregated on live, CD45+, TCRβ-, CD11b+ events (E) or on live, CD45+ events (G and I). Histological data are representative of two independent experiments (n=4-5 mice per group; A-D). Flow cytometric data is a representative of five independent experiments (n=4-5 per group; E-J). Percentages are shown as mean ± SEM.

To determine if the differences in frequencies of T cells in the lesions reflected differences in the blood, we analyzed T cells circulating in the blood of these animals at the same time post *L. major* infection. While a similar frequency and activation profile of CD4 T cells was observed between the two groups (Figure 3A), there was a significant increase in the percent of CD8 T cells circulating in the blood of LCMV immune *L. major* infected mice relative to control mice. This increase was due to an expansion in the effector memory population of CD8 T cells (Figure 2B) (Vezys et al., 2009). These results, taken together with our adoptive transfer experiments (Chapter 2, Figure 4), are consistent with the idea that LCMV memory CD8 T cells circulating in the blood are recruited into the leishmanial lesions.

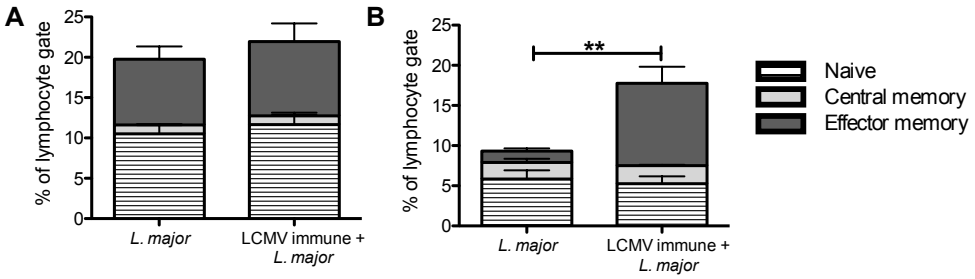

Figure 3. CD8 T cell population is significantly larger in LCMV immune mice while CD4 populations remain similar. B6 mice were infected with LCMV or left uninfected. After 30 days, mice were infected with metacyclic *L. major.* After 4 weeks, blood was taken from both groups and white blood cells were isolated. Cells were stained for surface T cell and activation markers. The proportion of CD4 T cells (A) or CD8 T cells (B) that were naïve ($CD44^{neg}$ $CD62L^{hi}$), central memory ($CD44^{hi}CD62L^{hi}$), or effector memory ($CD44^{hi}CD62L^{lo}$) was calculated as a frequency of cells in the lymphocyte gate. Data are representative of four independent experiments (n=5 per group).

CD8 T cells in leishmanial lesions express gzmB and markers of degranulation

Having seen a significant increase in the number of CD8 T cells within leishmanial lesions in LCMV immune mice, we next evaluated the function of these CD8 T cells. Cells were taken from the lesions of either control or LCMV immune mice and incubated

with BFA for 5 hours. We were particularly interested in examining the production of IFN-γ and gzmB, two of the major effector proteins of CD8 T cells. CD8 T cells from either group made little detectable IFN-γ (Figure 4A), and no detectable levels of TNF-α or IL-17 (data not shown). However, we found that the deficit in IFN-γ production was overcome if CD8 T cells were stimulated with PMA and ionomycin (data not shown), indicating that these cells were capable of making IFN-γ, but might not be receiving a sufficient stimulus to do so in the leishmanial lesions. In contrast to IFN-γ, the majority of CD8 T cells from the lesion express gzmB (Figure 4A). In addition, there were significantly more CD8 T cells in the LCMV immune *L. major* challenged mice expressing gzmB than in mice that received *L. major* alone (Figure 4B). CD8 T cells from the blood at the same time post-infection did not express either of these proteins (Figure 4C), suggesting that upregulation of gzmB was occurring within the lesion, although we cannot rule out the possibility that a small number of gzmB expressing CD8 T cells were preferentially recruited to the lesions.

Given the high expression of the cytotoxic protein gzmB, we wanted to assess whether CD8 T cells from the lesions were actively degranulating. CD107a is expressed in the cytotoxic granules and will be expressed on the surface of actively degranulating cells. To assess CD107a expression as a marker for degranulation, we added CD107a antibody to cultures of cells taken from the lesions that were incubated for 5 hours with BFA and monensin. There was detectable CD107a expression by CD8 T cells from the lesions of both control and LCMV immune mice, indicating that these CD8 T cells were not only expressing gzmB but were also actively degranulating (Figure 4A). As with the gzmB expression, there were significantly more CD107a expressing CD8 T cells in the LCMV immune lesions than in mice that had received *L. major* alone (Figure 4B).

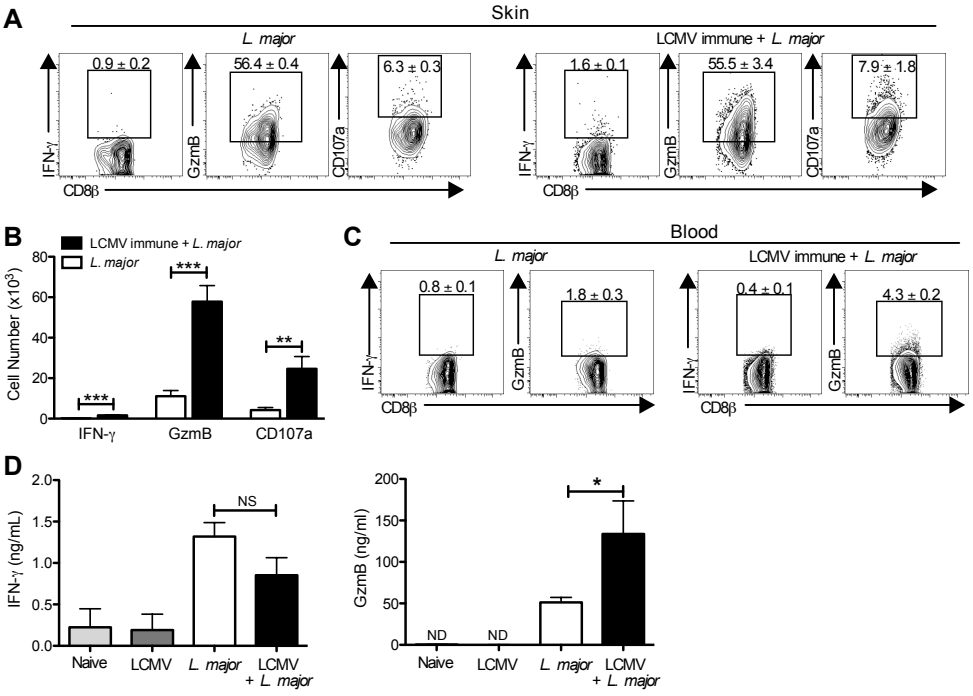

Figure 4. CD8 T cells infiltrating the leishmanial lesions express gzmB but low levels of IFN-γ. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, mice were infected with *L. major.* After 4 weeks, infected ears (A) and peripheral blood (C) were taken for analysis by flow cytometry. Cells from the infected ears were incubated with BFA, monensin and CD107a antibody for 5 hours and then stained for additional cell surface markers and intracellular proteins. Representative dot plots (A) and total cell numbers (B) are shown. Peripheral blood was taken and white blood cells were isolated and stained for cell surface markers and intracellular proteins. Representative dot plots are shown (C). Whole ear tissue was homogenized and supernatants were analyzed for gzmB and IFN-γ by ELISA (D). Flow data are representative of five independent experiments (n=4-5 mice per group). Ear supernatant data are representative of two independent experiments (n=4 mice per group). Percentages are shown as mean ± SEM.

Degranulation by NK cells in the lesions as measured by CD107a expression was not different in the two groups (data not shown).

The differential production of IFN-γ and gzmB by CD8 T cells was unexpected, and to directly determine whether the differences we saw by intracellular flow cytometry were reflected in the actual levels of IFN-γ and gzmB present in the lesions, we homogenized the lesions and collected the supernatants for analysis by ELISA. Consistent with the flow cytometry, similar levels of IFN-γ were detected in the lesions of LCMV immune *L.*

major infected and control mice (Figure 4D). Given that there were many more CD8 T cells present in the LCMV immune *L. major* infected lesions compared with controls, this result suggests that these CD8 T cells were not producing significant amounts of IFN-γ. In contrast, there was significantly more gzmB in supernatants from lesions taken from LCMV immune *L. major* infected mice compared to controls (Figure 4D). There was no detectable gzmB in supernatants taken from the skin of naïve mice or LCMV immune mice. These results support the flow cytometry data indicating that neither group of CD8 T cells make significant amounts of IFN-γ, while gzmB levels are significantly upregulated in LCMV immune *L. major* infected skin.

Finally, CD4 T cells from the lesions were also analyzed, and while they exhibited detectable levels IFN-γ, there were no differences seen between the two groups (data not shown). This data is consistent with what we saw upon restimulation responses to leishmanial antigen by draining lymph node cells and homogenized ear supernatants (Figures 1F and 4D). Given that CD4 T cell derived IFN-γ is crucial for controlling the parasites, this is also consistent with the data showing that the parasite burden of both groups were not different (Figure 1E).

LCMV specific CD8 T cells infiltrate leishmanial lesions

Given the increased number of CD8 T cells in the lesions of LCMV immune *L. major* infected mice, we hypothesized that this increase was due to an infiltration of LCMV specific CD8 T cells into the skin. To test this hypothesis we used two different approaches. First, we utilized a pool of 20 known LCMV peptides to restimulate cells taken from the infected ear, allowing us to broadly assess the LCMV responsiveness of the CD8 T cell population in the infected skin. Notably, a significant percentage of the CD8 T cells in the lesions were LCMV responsive, while there was a negligible response

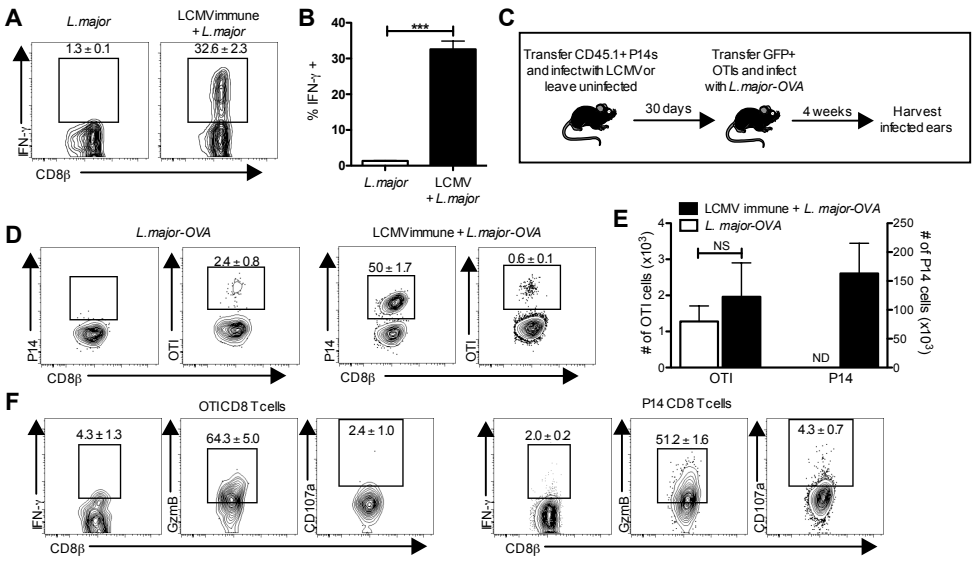

Figure 5. LCMV specific CD8 T cells are present in the leishmanial lesion and express gzmB but low levels of IFN-γ. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, mice were infected with *L. major.* After 4 weeks, *L. major* infected skin was taken and incubated with a pool of 20 LCMV derived peptides and BFA for 6 hours before staining for surface and intracellular proteins. Representative plots (A) and pooled data (B) are shown. Data are representative to 2 independent experiments (n=5 per group). P14 T cells were transferred into B6 mice and the next day a group was infected with LCMV. After 30 days, GFP+ OTI T cells were transferred into all groups and mice were infected with *L. major*-OVA (C). After 4 weeks, *L. major* infected skin was harvested and incubated with BFA for 5 hours. The samples were analyzed for the presence of P14 cells or OTI cells (D) and numbers of each were calculated (E). The OTI or P14 cells from the same infected ear of an LCMV immune *L. major* infected mouse were also incubated with BFA, monensin, and CD107a antibody then stained for additional surface and intracellular proteins (F). Data are representative of four independent experiments (n=4-5 per group). Percentages are shown as mean ± SEM.

by cells from mice infected with *L. major* alone (Figure 5A and 5B). This result indicates that a large proportion of the CD8 T cells that have been recruited to the leishmanial lesion are LCMV specific. In addition, these results further demonstrate that although CD8 T cells did not appear to be making IFN-γ within the leishmanial lesions (Figure 4A), when stimulated with LCMV peptides they were capable of producing IFN-γ.

We next used a transgenic T cell transfer system to allow us to directly identify LCMV specific and *L. major* specific T cells (Figure 5C). We transferred CD45 congenic P14 T cells into B6 mice that were subsequently infected with LCMV or left uninfected. After

thirty days, all mice received GFP^+ OTI CD8 T cells (OVA specific) and were challenged with *L. major* expressing OVA (*L. major*-OVA). The course of infection, increased lesion size in LCMV immune mice, and parasite control were all unchanged by the presence of these transgenic T cells (Figure 6A and 6B). After *L. major*-OVA infection, OTI cells were detectable in the lesions of both groups of mice, although at a relatively low percentage of the total CD8 T cells (Figure 5D and 5E). This most likely reflects the fact that many of the CD8 T cells recognize leishmanial antigens other than OVA. Of the CD8 T cells in the lesions of LCMV immune *L. major* infected mice, half were P14 cells (Figure 5D and 5E), once again confirming that a large proportion of the CD8 T cells present were LCMV specific memory cells. Also, despite *L. major*-OVA infected mice receiving the same number of P14 cells as all other groups, there was no detectable expansion of P14 cells in mice infected with *L. major*-OVA alone (Figure 5D and 5E).

LCMV specific cells express gzmB and markers of degranulation within leishmanial lesions

Using the transfer system described above, we found that neither P14 nor OTI cells from the lesions of LCMV immune *L. major* infected mice made much IFN-γ, suggesting that even *Leishmania*-specific CD8 T cells may be making little IFN-γ in the lesions during a primary infection (Figure 5F). In contrast, when gzmB expression was evaluated, the majority of both the OTI and P14 cells were gzmB positive (Figure 5F). Additionally, when we analyzed CD107a expression, there were detectable CD107a positive P14 cells. While the frequency of CD107a positive OTI and P14 cells was not significantly different, the number of CD107a positive P14 cells was substantially higher than OTI cells and included staining that is clearly above background levels (Figure 5F). Interestingly, in the blood neither IFN-γ nor gzmB were expressed by OTI or P14 cells

(Figure 6C), consistent with the idea that both antigen specific CD8 T cells and bystander CD8 T cells are induced to upregulate gzmB within the leishmanial lesions.

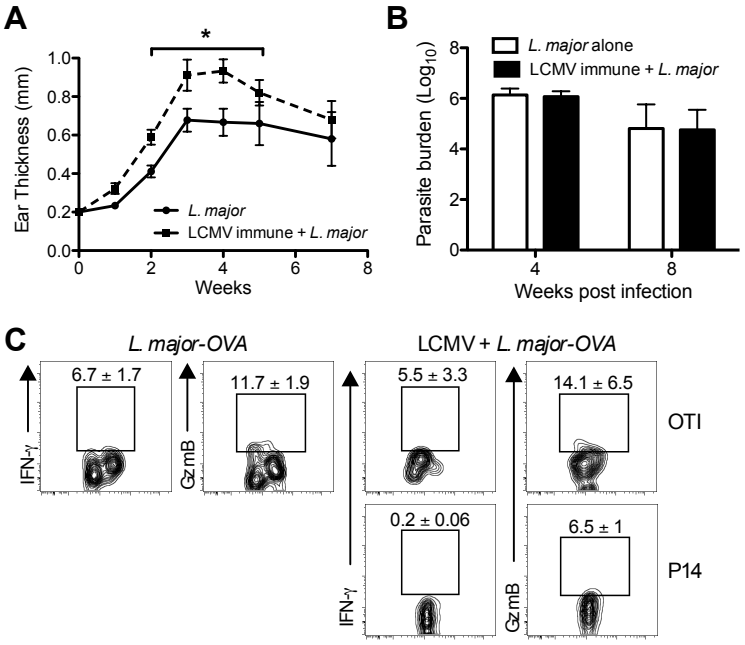

Figure 6. Transfer of P14 CD8 T cells and OTI T cells does not alter lesion progression or parasite control. P14 cells were transferred into B6 mice and the next day a group was infected with LCMV. After 30 days, GFP+ OTI cells were transferred and all mice were infected with *L. major*-OVA. Ear thickness was measured weekly (A). Infected skin was taken at various time points post infection and parasite burden was assessed using a limiting dilution assay (B). After 4 weeks, blood was taken from both groups and white blood cells were isolated. Cells were stained for surface and intracellular proteins (C). Data are representative of two independent experiments (n=4-5 per group). Percentages are shown as mean ± SEM.

No evidence for cross-reactivity between LCMV and *L. major*

In some studies, LCMV immune mice that are challenged with heterologous viruses have been shown to have overlapping or cross-reactive T cell responses (Brehm et al., 2002; Chen et al., 2012; Cornberg et al., 2010; Nie et al., 2010; Wlodarczyk et al., 2013). One way to detect these cross-reactive T cell populations is restimulation with LCMV peptides. Therefore, to test for cross reactivity of LCMV and *L. major* we stimulated cells

from the lesions of *L. major* infected mice with 20 different LCMV peptides. None of the LCMV peptides induced IFN-γ production in cells from *L. major* infected mice (Figure 5A and 5B). Since the hierarchy of immune dominant epitopes can be altered upon infection with a cross-reactive pathogen (Brehm et al., 2002), we also examined three of the top immunodominant epitopes for LCMV using tetramer staining. We found no change in the frequency of the LCMV epitopes in LCMV immune mice following *L. major* infection in either the blood or the spleen (Figure 7A and 7B). Moreover, tetramer staining of the spleen and blood from naïve mice or mice infected with *L. major* alone revealed similar frequencies of tetramer positive cells, indicating that *L. major* infection alone did not expand these three epitopes (Figure 7A and 7B).

In an alternative approach to look for cross-reactivity, we sorted polyclonal CD44hi CD8 T cells from LCMV immune mice, CFSE labeled them and transferred these cells into three groups: one that was left naïve, one that was then infected with *L. major*, and one that was then infected with LCMV. After 7 days, the transferred LCMV memory cells were detectable in the spleen and LN of all three groups, but only in the skin of LCMV infected recipients and not in the *L. major* infected skin (Figure 7C). Analysis of CFSE dilution and number of recovered cells revealed that LCMV memory cells transferred into *L. major* infected recipients did not proliferate above the background levels seen in naïve mice (Figure 7D and 7E). As expected, LCMV memory cells transferred into mice that were subsequently infected with LCMV all proliferated and expanded significantly in number (Figure 7D and 7E). Finally, since heterologous infections can cause shifts in the memory repertoire that results in impaired protective responses to rechallenge (Chen et al., 2012), we tested the ability of LCMV immune mice infected with *L. major* to handle the virulent LCMV clone 13 virus, and found that *L. major* infection had not functionally altered the ability to mount an effective LCMV immune response (data not shown).

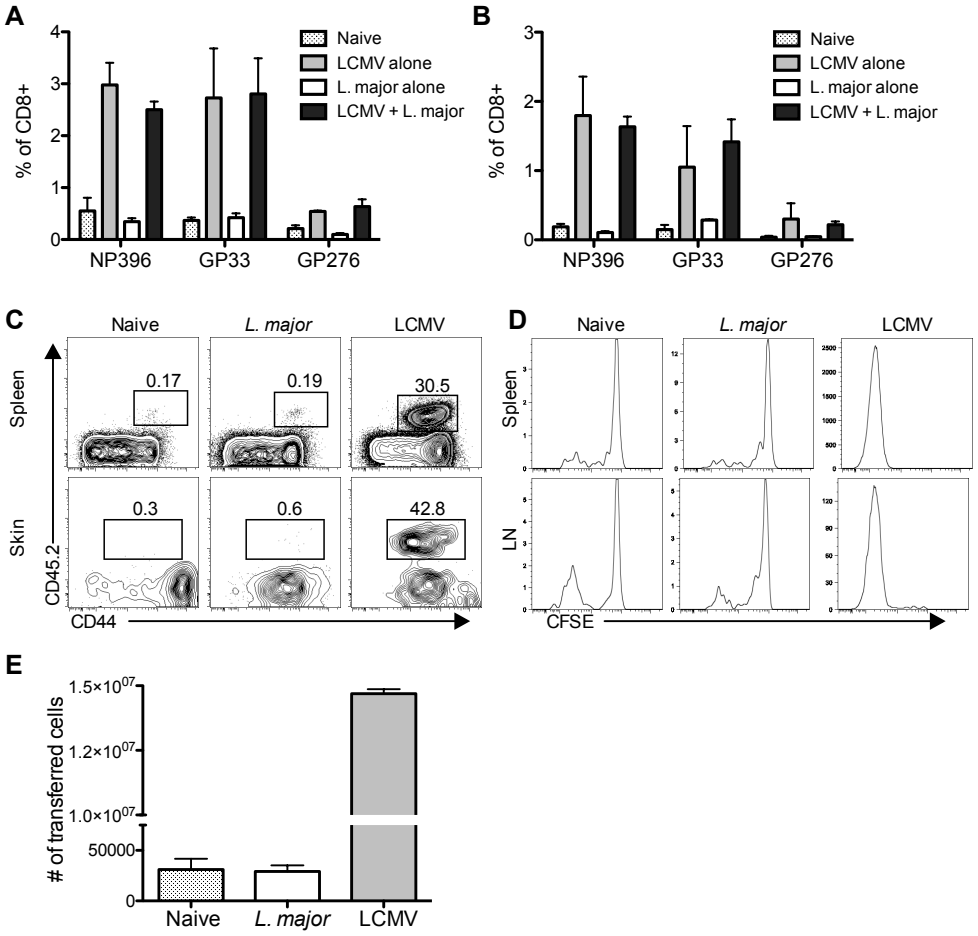

Figure 7. No evidence for cross-reactivity between LCMV and *L. major*. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, mice were infected with *L. major.* After 4 weeks, blood (A) and spleens (B) were taken for analysis by flow cytometry and stained with three different tetramers and other surface markers. B6 mice were infected with LCMV. After 30 days, splenocytes were harvested and $CD44^{hi}$ CD8 T cells were sorted. Equal numbers of $CD44^{hi}$ CD8 T cells were CFSE labeled and transferred into mice that were then left uninfected, infected with *L. major*, or infected with LCMV. After 1 week, spleens, skin and draining lymph nodes were harvested and transferred CD45.2+ cells were analyzed by flow cytometry. Representative plots (C and D) and pooled data (E) are shown. Data are representative of a single experiment (n=2-3 mice per group).

Taken together, these experiments indicate that there is no evidence that a cross-reactive epitope is responsible for the activation of LCMV-specific CD8 T cells in *L. major* lesions.

CD8 T cells required for increased pathology in *L. major* infected LCMV immune mice

Given the significant, prolonged infiltration of LCMV specific memory CD8 T cells into leishmanial lesions, we wanted to determine if the exacerbated immunopathology was dependent on these CD8 T cells. To test this, we took naïve or LCMV immune mice and treated them with depleting anti-CD8 antibodies for 2 weeks. Although depletion of memory CD8 T cells was slightly less effective than that of naïve CD8 T cells, the treatment significantly decreased the percentage of circulating CD8 T cells in the blood of LCMV immune mice prior to *L. major* infection (Figure 8A). We then infected the CD8 depleted and control mice with *L. major* and monitored the course of infection. Prior to infection with *L. major*, we ceased treatment with anti-CD8 antibodies and at 4 weeks post *L. major* infection the circulating levels of CD8 T cells in LCMV immune CD8 depleted mice had recovered to levels comparable with *L. major* infected non-depleted mice (Figure 8B). Consistent with previous studies, under these conditions CD8 depletion did not influence the course of *L. major* infection (Huber et al., 1998; Overath and Harbecke, 1993; Uzonna et al., 2004; Wang et al., 1993) (Figure 8C). As above, LCMV immune mice when challenged with *L. major* developed more severe lesions than control mice (Figure 8C). However, when CD8 depleted LCMV immune mice were infected with *L. major,* the lesion size and disease severity was similar to that seen in mice that had received *L. major* alone (Figure 8C and 8D). We found that the number of CD8 T cells in the lesions of LCMV immune CD8 depleted mice was similar to levels seen in mice that received *L. major* alone with no CD8 depletion, consistent with our

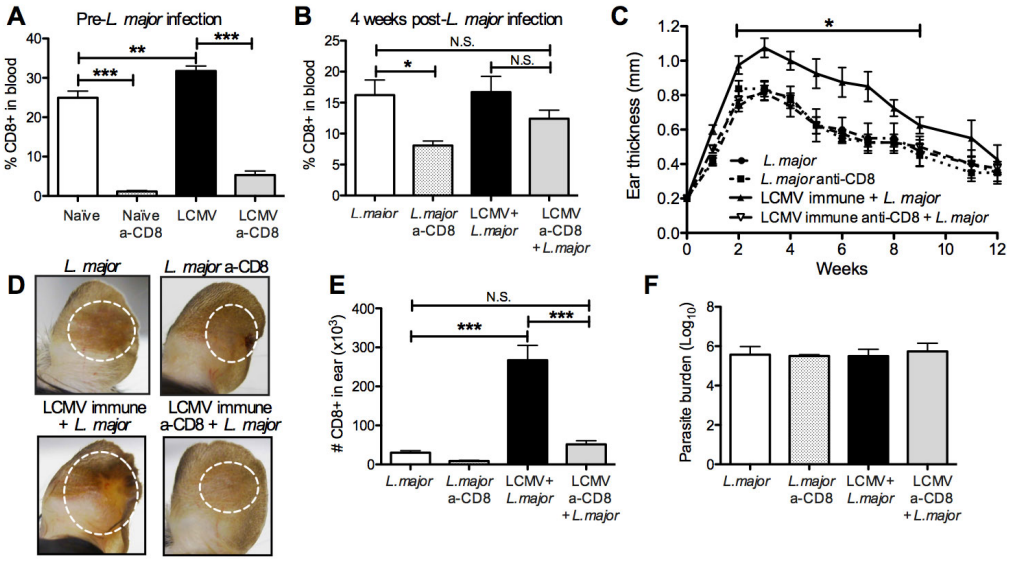

Figure 8. Exacerbated immunopathology is lost following depletion of CD8 T cells in LCMV immune mice prior to *L. major* infection. B6 mice were infected with LCMV or left uninfected for 45 days. Mice in each group were then treated with CD8-depleting antibody every 3 days for 15 days or left untreated. Blood was taken from animals in each group prior to *L. major* infection to assess CD8 depletion efficiency (A) and at 4 weeks post *L. major* infection to monitor reconstitution of the CD8 T cell compartment (B). All mice were with metacyclic *L. major* and ear thickness was measured weekly (C). Pictures were taken 4 weeks post *L. major* infection (D). Infected skin was taken at 4 weeks post infection and parasite burden was assessed using a limiting dilution assay (F). Infected skin was analyzed 4 weeks after *L. major* infection and the number of CD8 T cells present in each group was calculated (E). Data are representative of two independent experiments (n=10 per group).

ability to reduce the overall CD8 T cell population to normal levels (Figure 8E). In addition, the number of total cells infiltrating the LCMV immune CD8 depleted lesion was significantly reduced, reflecting a significant decrease in the number of monocytes and neutrophils present (data not shown). As observed above with LCMV immune mice, there was no change in the parasite burden in any of the groups (Figure 8F). Thus, these data clearly demonstrate that CD8 memory T cells are required for the increased immunopathology seen in LCMV immune mice.

CD8 T cells induce immunopathology in an NKG2D dependent manner

Having demonstrated that LCMV memory CD8 T cells are recruited and activated within leishmanial lesions, and that depletion of these cells abrogates increased disease, we further examined a mechanism by which the CD8 T cells were inducing immunopathology. In addition to expressing gzmB, LCMV memory CD8 T cells in leishmanial lesions of mice appeared to be degranulating, as measured by CD107a expression. This led us to examine alternate mechanisms for cytotoxicity by CD8 T cells that might be independent of cognate antigen interactions. A subset of memory CD8 T cells can express the activating NK cell receptor NKG2D. Engagement of this receptor can directly kill target cells, independent of TCR signals, through NKG2D recognition, as well as having a potent costimulatory effect on CD8 T cell activation (Groh et al., 2002; Jamieson et al., 2002; Meresse et al., 2004; Tietze et al., 2012; Verneris et al., 2004). There are several families of ligands for NKG2D and these ligands are all stress induced, MHC class I homologs (Champsaur and Lanier, 2010). We found that CD8 T cells in the lesions of *L. major* infected mice expressed low levels of NKG2D, but CD8 T cells from LCMV immune *L. major* infected lesions had significantly higher expression of NKG2D (Figure 9A). This increased expression, combined with the significantly larger number of total CD8 T cells present in the LCMV immune *L. major* lesions represents a substantial increase in the number of NKG2D positive CD8 T cells in these lesions. NK cells from the lesions of both groups had comparable levels of expression of NKG2D (data not shown).

Having seen increased expression of NKG2D on CD8 T cells, we next examined the lesions for expression of NKG2D ligands, specifically a member of the Rae1 family of ligands, Rae1-γ. Analyzing the skin by flow cytometry revealed expression of Rae1-γ by CD11b positive cells at a very low level in naïve skin. However, after infection with *L.*

major nearly all of the CD11b positive cells present in the lesion were expressing high levels of Rae1-γ (Figure 9B). Furthermore, analysis of Rae1-γ expression by immunohistochemistry demonstrated high levels of Rae1-γ within *L. major* lesions that are absent in naïve skin (Figure 9C). We observed expression of Rae1-γ both within the epidermis and widespread throughout the dermal layer of infected skin.

We next wanted to determine if we could link the levels of degranulation by CD8 T cells from the lesion to NKG2D engagement. To test this, we once again incubated cells from the lesions with anti-CD107a in the presence or absence of an antibody that blocks the interaction between NKG2D and its ligands. In the cultures where NKG2D interactions were blocked, there was significantly less CD107a staining in CD8 T cells from both groups (Figure 9D). These data indicate that NKG2D interactions contribute to the degranulation by CD8 T cells from the lesions.

NKG2D engagement can both be costimulatory or induce direct cytotoxicity in CD8 T cells (Zafirova et al., 2011). To determine whether the NKG2D expressing CD8 T cells from the lesions of LCMV immune mice were capable of directly lysing NKG2D ligand expressing cells, we used the VITAL in vitro killing assay (Hermans et al., 2004). Our target cells were RMA cells that expressed Rae1 or control RMA cells that did not express any NKG2D ligands (Cerwenka et al., 2001; Chu et al., 2013). We found that there was significantly more lysis of Rae1 expressing RMA cells than control RMA cells (Figure 9E). This could also be visualized by the selective loss of live Rae1 expressing RMA cells compared to control cells (Figure 9F). Taken together, these data suggest that CD8 T cells from the lesions of LCMV immune mice are capable of killing target cells in an NKG2D-dependent manner.

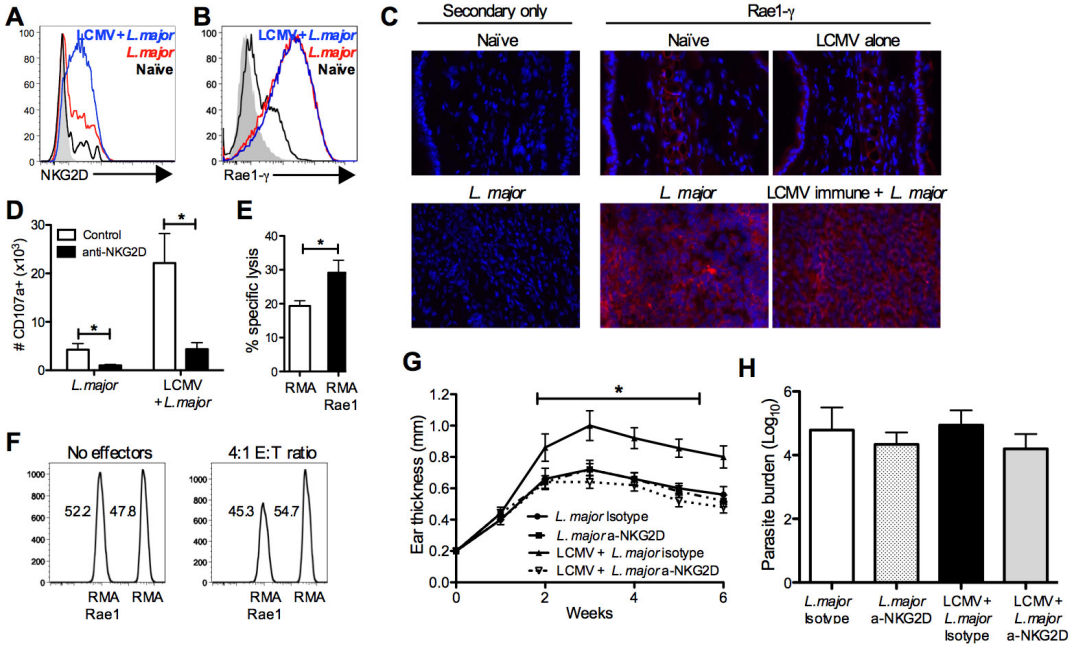

Figure 9. CD8 T cells induce immunopathology through engagement of NKG2D. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, mice were infected with *L. major.* After 4 weeks, infected skin was taken for analysis by flow cytometry (A, B and D) or frozen for immunohistochemical staining (C). Cells were pregated on live, CD45+, CD8β+ (A and D) or live, CD45+, CD11b+ (B). Frozen sections were cut and stained with DAPI (blue) and anti-Rae1-γ (red) and imaged at 40x magnification (C). CD8 T cells from the lesions of LCMV immune *L. major* infected mice were harvested 5 weeks post infection and enriched by negative selection. RMA cells were labeled with a high dose of CTV and Rae1 expressing RMA cells were labeled with a low dose for use as target cells. Control and Rae1 expressing cells were mixed 1:1 and incubated with LCMV immune CD8 T cells. Specific lysis was calculated for each group of target cells (E). The ratio of live target cells with or without effector cells is shown (F). Cells from the infected skin were divided and incubated with BFA, monensin, and CD107a antibody ±10 μg/ml of NKG2D blocking antibody and the number of CD107a+ cells calculated (D). B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, some mice in each group were treated with NKG2D blocking antibody or isotype control antibody. The following day mice were infected with *L. major*, and antibody treatment continued biweekly for the duration of the experiment. Ear thickness was measured weekly (G). Infected skin was taken 6 weeks post infection and parasite burden was assessed using an LDA (H). Data are representative of two independent experiments (n=5 per group; A-D and G-H) or a single experiment (n=5; E and F).

Ultimately we wanted to ascertain whether NKG2D expression by CD8 T cells was mediating the increased immunopathology observed in LCMV immune mice. We treated mice with NKG2D-blocking antibody or isotype control antibody starting one day before infection with *L. major* and twice a week after infection. We monitored lesion progression and found that while treatment with NKG2D-blocking antibody had no effect on the disease course in mice infected with *L. major* alone, it significantly reduced lesion size in LCMV immune mice infected with *L. major* (Figure 9G). As with CD8 depletion, despite completely reversing the increased immunopathology, there was no effect on parasite burden in any of the treatment groups (Figure 9H). Given that NKG2D is also an activating receptor on NK cells, we depleted NK cells during *L. major* infection and found that LCMV immune mice still exhibited exacerbated immunopathology (Figure 10). Taken together, these data demonstrate that LCMV memory CD8 T cells are recruited into leishmanial lesions, become activated, and induce immunopathology in an NKG2D dependent manner.

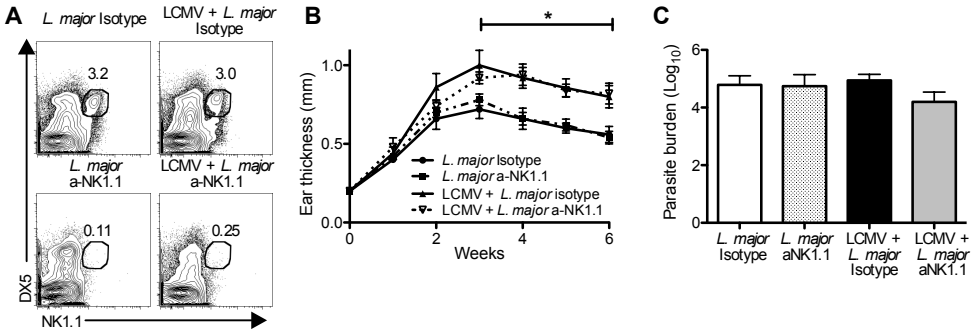

Figure 10. NK cell are not required for increased immunopathology in LCMV immune mice. B6 mice were infected with LCMV Armstrong or left uninfected. After 30 days, some mice in each group were treated with NK1.1 depleting antibody or isotype control antibody. The following day all mice were infected with *L. major*, and antibody treatment continued twice weekly for the duration of the experiment. Infected skin was taken at 6 weeks post infection and depletion of NK cells was assessed by flow cytometry (A). Ear thickness was measured weekly (B). Infected skin was taken 6 weeks post infection and parasite burden was assessed using an LDA (C). Data are representative of a single experiment (n=5 per group).

3.5 Discussion

This study demonstrates that memory CD8 T cells induced by a previous infection can enhance the disease severity of a subsequent unrelated infection in the skin. Thus, challenge of LCMV or *Listeria* immune mice with *L. major* resulted in increased lesion size and exacerbated pathology without any change in parasite control. Analysis of the leishmanial lesions in LCMV immune mice showed a greater than four-fold increase in the number of CD8 T cells, at least half of which were LCMV specific. We demonstrated that LCMV memory T cells can migrate into leishmanial lesions, and because LCMV immune mice maintained an elevated frequency of activated CD8 T cells circulating in the blood long after the clearance of virus (Vezys et al., 2009) we believe that the CD8 T cells in the lesions were from the blood. To test if these CD8 T cells were responsible for the increased pathology, LCMV immune mice were depleted of CD8 T cells prior to challenge with *L. major* and this depletion reversed the increased pathology observed in LCMV immune mice. Importantly, we further demonstrated that the CD8 T cells were meditating the increased pathology in an NKG2D dependent manner by blocking these interactions in vivo. Thus, our results show that bystander memory CD8 T cells can be recruited non-specifically to the skin during an infection-induced inflammatory response and instead of increasing protection, can exacerbate disease.

It has become increasingly clear that the study of a single pathogen in mice does not always reflect “real life” situations, where prevalent coinfections can have a significant impact on disease. For example, secondary bacterial infections are a common clinical complication during viral infections in the lung (Beadling and Slifka, 2004; Peltola and McCullers, 2004). Coinfection-induced changes in the host immune response can

contribute to disease progression by reducing the ability to control the secondary pathogen itself or to control pathogen-induced tissue damage (Jamieson et al., 2013; Jones et al., 1983; Okamoto et al., 2003). In addition to acute coinfections, chronic infections, such as helminths or mycobacteria, significantly impact the immune responses to other infections or vaccines (reviewed in Stelekati and Wherry, 2012). In leishmaniasis, HIV coinfection contributes to enhanced disease, as does simultaneous infection with schistosomes (Andreani et al., 2012; La Flamme et al., 2002). Taken together these studies argue that fully understanding the outcome of one infection may require knowledge about additional ongoing infections. Our study expands this concept to include the influence of previous infections. Thus, while it is clear that secondary infections occur and pose a significant health risk, every human has circulating memory T cells that could potentially impact the disease outcome for future infections. This makes understanding the role of bystander T cells in disease an important and distinct question for understanding human disease.

While it has been more than 20 years since bystander T cells were first hypothesized to participate in immune responses, their relative contribution to the immune response remains controversial (Ehl et al., 1997; Masopust et al., 2007; Murali-Krishna et al., 1998; Tough et al., 1996). In the context of viral infections, bystander T cell effects are often attributed to cross-reactivity and may therefore be TCR-driven (Chen et al., 2001; Chen et al., 2003; Kim et al., 2002; Selin et al., 1998). While cross-reactive peptides have been identified for some virus pairs, it is a technically challenging endeavor and in many cases it is still unclear which epitopes are cross-reactive (Brehm et al., 2002; Chen et al., 2012; Cornberg et al., 2010; Mathurin et al., 2009; Nie et al., 2010; Welsh et al., 2004; Wlodarczyk et al., 2013). However, the recent use of TCR transgenic T cells has provided convincing results that memory CD8 T cells can be activated by inflammatory

cytokines and contribute to immune responses without the need for cognate antigen stimulation (Berg et al., 2003; Freeman et al., 2012; Kohlmeier et al., 2007; Kohlmeier et al., 2010; Soudja et al., 2012; Tough et al., 1996). For example, protective immunity to *Listeria* can be generated using TCR transgenic memory cells even when the challenging strain of *Listeria* does not express the cognate antigen recognized by the transgenic T cells (Berg et al., 2003; Soudja et al., 2012). IFN-γ production and proliferation of memory cells are two major readouts that have been used to demonstrate cross-reactivity (Chen et al., 2001; Kim et al., 2005; Selin et al., 1998; Wlodarczyk et al., 2013), and LCMV memory cells did not produce IFN-γ in response to restimulation with leishmanial antigen and did not proliferate in response to infection with *L. major* (Figure 1F and 7). Changes in epitope dominance are also an indication that cross-reactivity is playing role (Brehm et al., 2002), however there was no change in the dominance hierarchy of major LCMV epitopes following *L. major* infection and no expansion of these epitopes was observed in animals infected with *L. major* alone (Figure 7A and 7B). Moreover, despite *L. major* infected animals receiving the same number of P14 transgenic T cells prior to infection as the LCMV immune group, there was no expansion or activation of these cells in response to *L. major* infection alone. Yet a majority of the P14s present within the lesions of LCMV immune *L. major* infected mice were activated and expressing gzmB (Figure 5D and 5E). Additionally, we observed the same phenotype and activation of CD8 T cells from *Listeria* immune animals during *L. major* infection (Figure 1 and data not shown). Overall, while the complexity of these pathogens leaves open the possibility of some undetected cross-reactivity being involved in the responses we have observed, all of our results are consistent with activation of bystander CD8 T cells due to the inflammatory milieu present in the lesion.

A key difference between many of the previous studies of bystander T cell responses and our current findings is that instead of increased protection, we find that bystander memory CD8 T cells have no impact on parasite control, but instead increase pathology. As most studies have monitored the response of bystander T cells during acute infections (Berg et al., 2003; Chen et al., 2001; Selin et al., 1998; Soudja et al., 2012), it is possible that activation of bystander T cells in a chronic, inflammatory environment is responsible for these differential effects. The sustained recruitment of cells over many weeks in leishmaniasis may lead to a greater accumulation of bystander CD8 T cells that can promote pathology. Our study is also the first to characterize the role of bystander CD8 T cells in the skin, where the pathologic effects of the bystander CD8 T cell activation can be directly visualized. The most striking difference between our results and prior studies is that following *L. major* infection the bystander CD8 T cells recruited to the skin made gzmB, but little IFN-γ. This is in contrast to other infections where bystander CD8 T cells were activated and quickly produced IFN-γ, to provide increased protection (Chen et al., 2001; Chen et al., 2003; Selin et al., 1998; Soudja et al., 2012). What accounts for this difference in effector function is unclear, however it may be that the inflammatory response induced by *L. major* is insufficient to promote IFN-γ production. Different cytokine environments can differentially regulate the effector profile of CD8 T cells, with cytokines like IL-12 and IL-18 inducing high amounts of IFN-γ, while IL-15 induces the upregulation of gzmB (Freeman et al., 2012; Kohlmeier et al., 2010; Raué et al., 2013; Soudja et al., 2012; Varadarajan et al., 2011). We are currently examining the role of these cytokines on CD8 T cell activation within leishmanial lesions. Overall, our work suggests that the nature of the inflammation, including duration, location, and composition, will impact whether bystander CD8 T cell activation will be protective or pathologic.

CD8 T cells have many effector functions that could be playing a role in pathogenicity. For example, CD8 T cell derived CCL3 and CCL4 increases recruitment and activation of the inflammatory cells into tissues (Narni-Mancinelli et al., 2007). Production of chemokines by tissue resident memory CD8 T cells is also important for recruitment of CD8 T cells to sites of inflammation (Schenkel et al., 2013). Similarly, CD8 T cells produce inflammatory cytokines, such as IFN-γ, TNF-α, and IL-17 that can promote tissue destruction (Ortega et al., 2009; Res et al., 2010; Wherry et al., 2003). However, the levels of these particular cytokines were low or absent in CD8 T cells within the leishmanial lesions, making them unlikely to be playing a major role (Figures 4 and 5 and data not shown). Additionally, large changes in IFN-γ production would most likely result in increased parasite killing, which was not observed (Figure 1E). While bystander CD8 T cells would not be expected to exhibit antigen-specific cytolytic activity, we have demonstrated that LCMV specific CD8 T cells can act non-specifically through an NKG2D-dependent mechanism (Figure 9). Recent work has shown that NKG2D engagement on bystander CD8 memory cells can serve as an innate-like killing mechanism for early control of bacterial burdens in *Listeria* infection (Chu et al., 2013). Here we highlight the pathologic role this pathway can play, leading to excessive cell death and the induction of enhanced inflammation. These experiments emphasize an underappreciated effector function of CD8 T cells that can have serious consequences for disease progression in leishmaniasis, and potentially other diseases as well.

The formation of a protective memory T cell pool is a crucial part of an effective immune response. However, unlike highly controlled mouse models of disease, humans encounter many pathogens throughout their life that can increase the size and diversity of their immunological memory pool. Determining how this pool of memory T cells impacts future immune responses to unrelated pathogens may be critical for

understanding the diversity observed in human disease. While differences in each individual's response to a given infection is related to pathogen dose and the genetic and physiological status of the host, our work suggests that the immunological history of a patient may also play a role. *Leishmania* infections have a very broad spectrum of clinical disease ranging from subclinical infection to ulcerating skin lesions to destruction of the mucosal tissue (Lessa et al., 2007; Murray et al., 2005; Pearson and Sousa, 1996). Both parasite and host factors have been implicated in this diversity, but our current work indicates that the immunological history of the patient may also directly impact the severity of leishmanial disease. In support of this idea, non-*Leishmania* specific CD8 T cells are found within human leishmanial lesions (Da-Cruz et al., 2010) and there is a correlation between the number of intralesional CD8 T cells and disease severity (Faria et al., 2009; Santos et al., 2013). Taken together, our studies demonstrate that bystander CD8 T cell activation can play a role in leishmanial lesion progression through the engagement of an NK cell receptor, NKG2D. On a broader level, our studies highlight the potential importance of heterologous immunological responses, which remains an under-appreciated aspect of the host immune response to infection. Understanding how prior immune responses influence subsequent infections and infection-induced pathology will be important for designing new and effective therapeutic treatments. This work has identified NKG2D and its ligands as potential therapeutic targets in leishmaniasis and has broad implications for potential role of this pathway in other infections where excessive tissue damage and immunopathology are observed.

Chapter 4 Coinfection with LCMV exacerbates lesion progression and parasite control in *Leishmania major* infected mice in an NKG2D dependent manner

4.1 Abstract

Leishmania is a significant neglected tropical disease that infects 1-2 million people every year and causes a life long persistent infection. Due to both the chronic nature of this parasite and its overlapping geographical distribution with many other significant diseases, including many species of intestinal helminths and HIV, there is a high incidence of coinfection occurring within patients. Analysis of CD8 T cells isolated from the lesions of patients revealed that many appear to be specific for pathogens other than *Leishmania*. Despite this, little work has been done to evaluate the impact of coinfections on *Leishmania* disease progression. Here we show that infection with the acute virus, lymphocytic choriomeningitis virus (LCMV), during active *Leishmania major* (*L. major*) infection results in significantly increased disease severity and elevated parasite burden at the peak of infection. Despite this dramatic change in *L. major* progression, LCMV was cleared normally. This coinfection resulted in increased infiltration of CD4 and CD8 T cells as well as a massive influx of neutrophils. CD8 T cells present in the lesions of coinfected mice expressed high levels of gzmB and the NK cell receptor NKG2D. Treatment of mice following infection with LCMV with NKG2D blocking antibody completely reversed the increase in both disease severity and parasite burden. Together this study shows that during a coinfection, engagement of NKG2D can exacerbate disease progression and suggests that targeting of this pathway may be an option to control immunopathology in *Leishmania* infected patients.

4.2 Introduction

Chronic infections with persistent pathogens impact more than a third of the world's population. One such infection is caused by the protozoan parasite *Leishmania*, which infects 1.2 million people every year and is responsible for the ninth largest disease burden among individual infectious diseases (Alvar et al., 2012). This neglected tropical disease has many diverse clinical manifestations from ulcerative skin lesions to disseminated visceral infection. Approximately 90% of all *Leishmania* infections are localized cutaneous forms, but even within cutaneous infections there is a wide spectrum of disease severity (World Health Organization, 2014). Many factors likely play a role in this disease spectrum including host and parasite factors, but the specific causes are unclear.

Experimental mouse models have demonstrated that control of *Leishmania* infection requires a robust Type I immune response, characterized by IFN-γ production to activate infected cells and kill intracellular parasites (Sacks and Noben-Trauth, 2002). The chronic nature of *Leishmania* infection and the geographic regions in which it is endemic, predispose infected patients to a wide variety of coinfections. Many species of intestinal helminths are prevalent in regions of the world where *Leishmania* infection occurs and a study of coinfected individuals revealed that the presence of a helminth resulted in slower healing and increased Type II immune responses as measured by IgE levels (O'Neal et al., 2007). Mouse models of coinfection with *Schistosoma mansoni* and *Leishmania* showed a similar skewing towards a Type II immune response with increased levels of IL-4 production and subsequently increased parasite burden and delayed lesion resolution (La Flamme et al., 2002). Given this, it follows that a Type I inducing coinfection may prove to be protective, and in fact, susceptible mouse strains can be protected when mice are coinfected with *Leishmania* and *Toxoplasma gondii* (*T.*

gondii) or *Listeria monocytogenes* (*Listeria*) (Santiago et al., 1999; Tabbara et al., 2004). However, lesion size and severity are not always associated with high parasite burden. The most severe form of cutaneous infection, where mucosal tissue is destroyed, is associated with relatively low levels of parasite and an overly exuberant immune response including high levels of pro-inflammatory cytokines (Bacellar et al., 2009).

Here we demonstrate that coinfection of *L. major* infected mice with a Type I inducing acute virus, Lymphocytic Choriomeningitis virus (LCMV), not only fails to protect coinfected mice but rather exacerbates disease severity and increases parasite burden. Surprisingly, this increased disease is not associated with increased levels of IFN-γ or IL-17 in the lesion, but rather an influx of gzmB expressing LCMV specific CD8 effector cells. Both the immunopathology and increase in parasite burden could be blocked by treatment with anti-NKG2D, an NK cell receptor expressed by the infiltrating CD8 T cells. This work highlights the fine balance between controlling infection and inducing immunopathology and demonstrates that this balance can be disrupted by an unrelated infection.

4.3 Materials and methods

Animals

Female C57BL/6 mice (6 weeks old) were purchased from the National Cancer Institute (Fredericksburg, MD). Animals were housed in a specific pathogen-free environment and tested negative for pathogens in routine screening. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee, University of Pennsylvania Animal Welfare Assurance Number A3079-01.

Parasite, virus, and bacterial infections

L. major parasites (Friedlin) were grown to the stationary phase in Schneider's Drosophila medium (Gibco) supplemented with 20% heat-inactivated FBS (Gibco) and 2 mM L-glutamine (Sigma) at 26°C (Prickett et al., 2006). Metacyclic promastigotes were isolated from 4-5 day old stationary cultures by density gradients (Späth and Beverley, 2001). Mice were infected with 2 x 10^6 metacyclic parasites injected intradermally into the ear. Lesion development was monitored weekly by taking measurements of ear thickness with digital calipers (Fisher Scientific). Parasite burden in lesion tissues was assessed using a limiting dilution assay as previously described (Scott et al., 2004). For viral infections, mice were infected with 2 x 10^5 PFU of LCMV Armstrong strain by i.p. injection.

Flow cytometry

For flow cytometry, cells were isolated from ears, draining lymph nodes, spleens or peripheral blood. For ears, dermal sheets were separated and incubated in incomplete IMDM+GlutaMAX (Gibco) containing 0.25 µg/mL of Liberase TL (Roche, Diagnostics Corp.) and 10 µg/mL DNase I (Sigma-Aldrich) for 90 minutes at 37°C. Ears, draining lymph nodes, and spleens were mechanically dissociated by smashing through a 40-µm cell strainer (Falcon) in PBS containing 0.05% BSA and 20 µM EDTA. Splenocytes were incubated for <1 minute with ACK lysing buffer (Lonza) to lyse red blood cells. When indicated, cells were incubated at 4 x 10^6 cells/ml with Brefeldin A (BFA, 3 µg/ml final concentration) (eBioscience) alone for 5 hours before staining for flow cytometry. Cells were then incubated with FC block (anti-CD16/32, heat inactivated mouse sera and Rat IgG) followed by fluorochrome-conjugated antibodies for surface markers CD45, CD8β, CD4, CD44, CD62L, CD11b, Ly6C, and/or Ly6G (1A8) (all eBioscience) and were

fixed with 2% paraformaldehyde (Electron Microscopy Sciences). Tetramers for GP33 and NP396 conjugated to APC were used where indicated. For intracellular staining, cells were previously permeabilized with 0.2% of saponin buffer and stained for IFN-γ, gzmB, TNF-α, and/or IL-17A (eBioscience or Invitrogen). The data were collected using an LSR Fortessa flow cytometer (BD Bioscience) and analyzed with FlowJo software (Tree Star).

Histopaque

Peripheral blood was collected into a 4% sodium citrate solution. White blood cells were isolated by underlaying with Histopaque-1083 (Sigma) and spinning for 20 minutes at 400 x g at room temperature.

Ear Homogenization

Whole ears were placed in ice cold PBS with a protease inhibitor cocktail (Sigma). Samples were homogenized using the FastPrep-24 (MP Biomedicals) and spun for 5 minutes at 5000 rpm at 4°C in a microcentrifuge. The supernatants were removed and stored at -80°C until analysis by ELISA as described below.

Leishmanial antigen restimulation and ELISAs

Leishmanial antigen was obtained from stationary-phase promastigotes of L. major by resuspending parasites at 1 x 10^9 parasites/ml in PBS and conducting 20 freeze/thaw cycles. For measurements of antigen-specific cytokine production, the infected skin draining retroauricular lymph node was removed, mechanically dissociated, and single cell suspensions were prepared. Cells were resuspended in complete IMDM+GlutaMAX (Gibco) supplemented with 10% heat inactivated FBS (Gibco), 2 mM L-glutamine (Sigma), 100 U of penicillin and 100 μg of streptomycin (Sigma) per mL and 0.05 μM of

β-mercaptoethanol (Sigma). Cells were plated at 4 x 10^6 cells/mL in 1 ml in 48-well plates. Cells were incubated at 37°C in 5% CO_2 with 20 x 10^6 L. major parasites/mL. Supernatants were collected after 72 hours and stored at -20°C until they were assayed by sandwich ELISA using paired monoclonal antibody to detect IFN-γ, IL-4 or IL-17 (eBioscience). Cytokine concentrations were calculated from standard curves with detection limit of 0.03 ng/mL for IFN-γ, 0.015 ng/mL for IL-17A and 7 Units/mL of IL-4. Granzyme B was analyzed by ELISA using a mouse granzyme B Duoset kit (R&D Systems). IL-1β was analyzed by ELISA using a mouse IL-1β ELISA MAX kit (Biolegend).

In vivo antibody treatment

NKG2D blocking antibodies (200 µg/dose; Clone HMG2D; BioXCell) were given intra-peritoneal 3 days after infection with LCMV and twice weekly for the duration of the experiment.

Statistics

Results represent means ± SEM. Data were analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined using unpaired, one-tailed Student's *t* test with p values given as: *p < 0.05; **p < 0.001; and ***p < 0.0001; ns p > 0.05. Results with a p value ≤0.05 were considered significant.

4.4 Results

Coinfection with LCMV exacerbates lesion formation and increases parasite burden in *L. major* infected mice

Previous work has focused on the role of immunological memory on the progression of disease during *L. major* infection (Chapter 3). Given that *Leishmania* is a chronic infection and lesions can persist for several months, we also wanted to investigate the

impact of an unrelated infection on the disease course of an established leishmanial lesion. We infected mice intradermally with *L. major* and waited 2 weeks for a measurable lesion to form. A cohort of these mice was then infected with LCMV Armstrong and the disease progression was followed for 13 weeks with ear measurements taken weekly (Figure 1A). Mice coinfected with LCMV had a significant increase in lesion size and delayed healing time following infection with LCMV. Photographs taken at the peak of lesion formation highlight the increased tissue damage and ulceration that occurs in the coinfected mice (Figure 1B), but even more striking is the structural damage and tissue loss that remains even after inflammation and ulceration have resolved (Figure 1C). Interestingly, the increased pathology is associated with increased parasite burden in the skin at 3 and 5 weeks post infection (Figure 1D) and 5 weeks post infection in the DLN (Figure 1E). Analysis of the skin at 7 weeks post infection seems to indicate that the parasite burden is controlled similarly in the coinfected mice, but later time points are needed to definitively determine this. This finding is in contrast to that seen with a previous infection with LCMV, in which pathology was not associated with any concomitant change in parasite control (Chapter 3).

Coinfection with LCMV inhibits *Leishmania* specific IFN-γ production

Having seen an increase in parasite burden in the coinfected mice, we examined the anti-leishmanial immune response to determine if it was altered upon infection with LCMV. Mice were once again infected with *L. major* and at 2 weeks post infection half of them were challenged with LCMV. At 3 weeks post *L. major* infection, cells were isolated from the DLN of infected mice, including age matched naïve controls and age and infection matched LCMV alone controls. These lymph node cells were cultured for 72 hours with leishmanial antigen (FTAg) or media alone. At the end of the culture, supernatants were removed and fresh media with BFA was added for the final 6 hours of

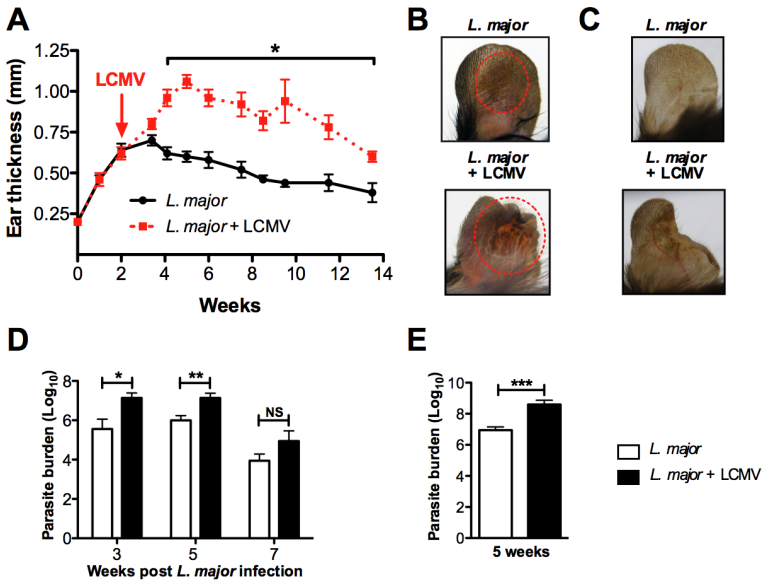

Figure 1 Coinfection of *L. major* infected mice with LCMV exacerbates lesion formation and leads to an increase in parasite burden. Mice were infected with *L. major* or left uninfected for 2 weeks. Some mice from each group were then infected with LCMV and ear thickness was measured weekly (A). Lesions were photographed at 5 weeks (B) or 15 weeks (C) post *L. major* infection. Parasite burden was determined using an LDA at 3, 5, and 7 weeks post *L. major* infection in the skin (D) and at 5 weeks post *L. major* infection in the DLN (E). Data are representative of 4 independent experiments (n=4-5 mice per group). Error bars represent SEM.

culture. Cells were then stained and analyzed for IFN-γ production (Figure 2A). As expected, neither CD4 nor CD8 T cells from naïve or control LCMV infected mice produced IFN-γ in response to FTAg stimulation (Figure 2A-2D). Mice infected with *L. major* alone produced IFN-γ specifically in response to stimulation with FTAg (Figure 2A-D). This IFN-γ production was detected intracellularly by cytokine staining as well as extracellularly in the supernatants by ELISA (Figure 2A and 2D). Interestingly, cells taken from coinfected mice had a defect in FTAg induced IFN-γ production (Figure 2A-2C). This was reflected by a decrease in the percentage of CD4 and CD8 T cells staining for IFN-γ intracellularly as well as a decrease in the total accumulated IFN-γ found in the supernatants (Figure 2A and 2D).

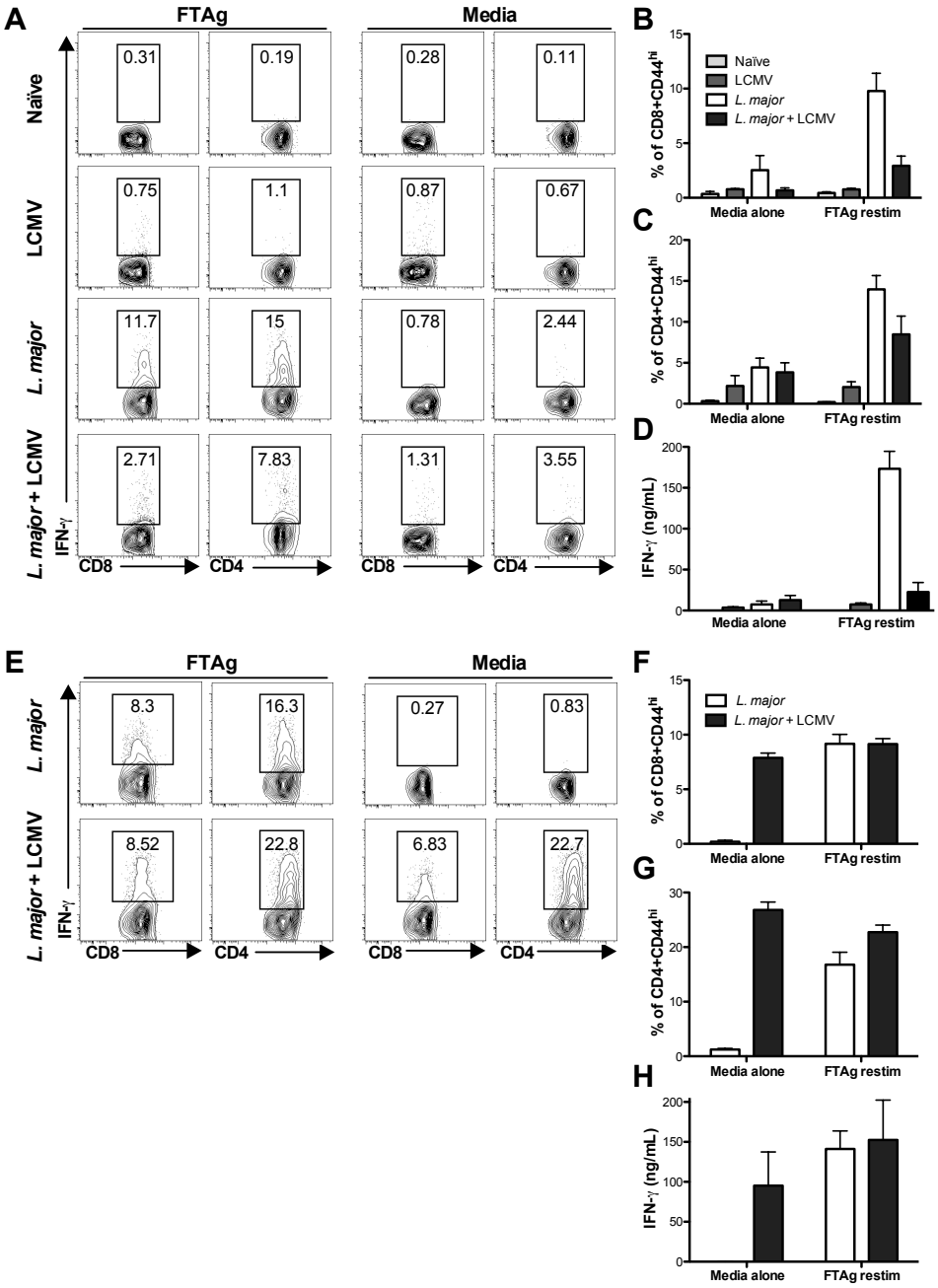

Figure 2 Coinfection with LCMV inhibits leishmania specific IFN-γ production early. Mice were infected with *L. major* or left uninfected for 2 weeks. Some mice from each group were then infected with LCMV. Cells from the DLN of infected mice or pooled LNs from naïve mice were stimulated in vitro with media alone or leishmanial antigen (FTAg) 1 week (A-D) or 3 weeks (E-H) after LCMV infection for 72 hours. After 72 hours, supernatants from the cultures were removed and analyzed by ELISA (D and H). BFA and fresh media were added to the cells for 6 hours, after which they were stained for IFN-γ (A and E). The percentage of CD44hi CD8 (B and F) and CD4 (C and G) T cells making IFN-γ was calculated. Data are representative of two independent experiments (n=3-5 mice per group). Error bars represent SEM.

There are several possible explanations for the IFN-γ defect that was observed. It is possible that the decrease in *Leishmania* responsive cells in the DLN is due to the significant expansion of LCMV specific cells at this time point (Masopust et al., 2007) leading to a decreased number of *L. major* specific cells in the same number of total LN cells. The defect in IFN-γ production could also be the result of a temporary anergic state seen in T cells following acute infection with LCMV, during which cells are refractory to additional stimulation (Brojorge.K and Volkert, 1974; Butz and Southern, 1994; Mims and Wainwrig, 1968; Saron et al., 1990). High levels of Type I IFNs can also induce this unresponsive state (Marshall et al., 2011; Redford et al., 2014; Wilson et al., 2013). Regardless of the mechanism involved, given the critical role that IFN-γ plays in controlling parasite burden, an impairment of *Leishmania* specific IFN-γ production is consistent with the increased parasite burden observed in the skin and DLN early on during the coinfection (Figure 1C and 1D).

If the loss of functionality observed above was dependent on LCMV infection induced unresponsiveness, we would predict that it would be transient, and should responsiveness to *Leishmania* antigen should return following resolution of LCMV associated inflammation. Therefore, similar analysis of DLN cells from mice 5 weeks post *L. major* infection. At this time point, IFN-γ production by both CD4 and CD8 T cells in coinfected mice has recovered to levels similar to those seen in mice infected with *L. major* alone (Figure 2E-2H). Also contrary to what was seen at 3 weeks post *L. major* infection, cells from coinfected DLNs 5 weeks post *L. major* infection produced comparable levels of IFN-γ regardless of whether FTAg was added to the cultures or not (Figure 2E). This is observed in both the flow analysis of intracellular IFN-γ and the ELISA for IFN-γ protein in the supernatants (Figure 2E and 2H). The recovery of IFN-γ production is also consistent with the finding that parasite burdens in the coinfected mice

are controlled at later time points similarly to *L. major* singly infected animals (Figure 1C). The increased background IFN-γ production seen in unstimulated DLN cells from coinfected mice may be due to the increased parasite load present in this tissue at this time point, stimulating IFN-γ production even in absence of additional exogenous leishmanial FTAg (Figure 1D). Future work will determine if this is a general IFN-γ overproduction that occurs at this time post LCMV infection or an attribute that is specific in our coinfected mice.

Coinfection does not alter clearance of LCMV or the expansion of LCMV specific CD8 T cells

Given the impact of LCMV infection on the control of *L. major*, we wanted to investigate whether coinfection altered the ability to respond to or control LCMV. Serum, spleen, and kidney samples were taken from mice infected with LCMV alone or coinfected mice at 3, 7 and 10 days post LCMV infection. These tissues were analyzed for LCMV viral titers and virus was detectable at similar levels between the two groups in the serum, kidney, and spleen at day 3 post LCMV infection (Figure 3A). The virus was not detectable in any of the tissues from either group by day 7 or 10 post LCMV infection, indicating that the virus is controlled similarly regardless of whether an *L. major* infection is present.

Another measure of the LCMV immune response is to track expansion of the CD8 T cells. Analysis of the activated population of CD8 T cells in the spleen at 7 days post infection as measured by their expression of CD44 and CD62L revealed similar activation profiles in LCMV infected mice irrespective of *L. major* infection (Figure 3B). This included significant expansion of activated CD8 T cells, with the CD44hi population accounting for almost 100% of CD8 T cells present in the spleen (Figure 3B). This is in

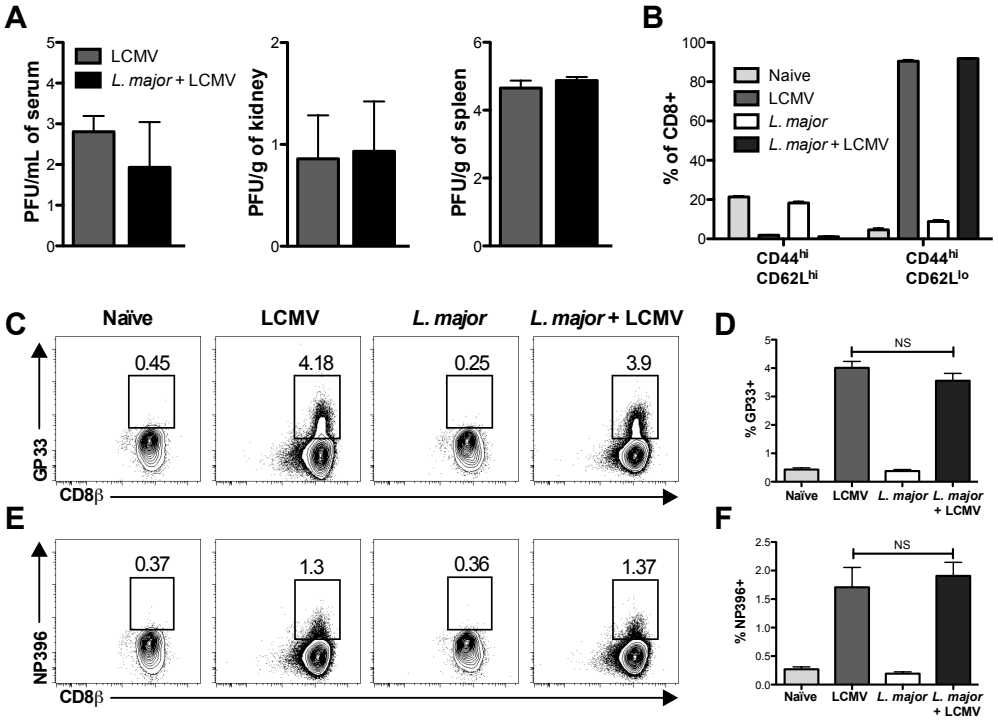

Figure 3 Coinfection of LCMV and L. major does not alter the magnitude of the LCMV immune response or the clearance of virus. Mice were infected with *L. major* or left uninfected for 2 weeks. Some mice from each group were then infected with LCMV. Three days after infection with LCMV, serum, kidney, and spleen samples were taken to assess viral titers by plaque assay (A). One week post infection with LCMV, spleens were harvested and stained with activation markers CD44 and CD62L (B). Quantification of each cell population is shown as a percentage of total CD8+ cells. These splenocytes were also stained with LCMV tetramers, GP33 (A) and NP396 (C). Tetramer plots were pregated on live/CD8+/CD44^{hi} cells. Representative plots (C and D) and quantification (D and F) are shown. Data is representative of a single experiment (n=3-4 mice per group). Error bars represent SEM. NS = not significant

contrast to the relatively low proportion of activated CD8 T cells in mice infected with *L. major* alone. Staining with tetramers for two of the immunodominant LCMV epitopes also showed similar frequencies of GP33+ and NP396+ CD8 T cells in LCMV alone and coinfected mice (Figure 3C-3F). Taken together, these data demonstrate that clearance of the virus and the magnitude of the immune response at the peak are unaffected by *L. major* infection.

Coinfection significantly alters the cellular infiltration into *L. major* infected skin

Coinfection with LCMV after the establishment of a *L. major* infection induced significantly larger lesions characterized by increased ulceration and tissue loss. Given this pathology, we next wanted to examine the composition of cells present in these lesions. The peak of lesion formation was 5 weeks post *L. major* infection and 3 weeks post LCMV infection. At this time, we digested the infected skin and analyzed the cellular infiltration by flow cytometry. As predicted based on our previous work with LCMV effector cell transfers and infection of LCMV immune mice (Chapters 2 and 3), there was a significant increase in the frequency of CD8 T cells present in the lesions of coinfected mice (Figure 4A-4B). In our previous work with LCMV immune mice, despite an increase in CD8 T cells, there was no change in CD4 T cell infiltration into the lesions (Chapter 3). Additionally, while the number of monocytes and neutrophils also increased in LCMV immune *L. major* infected mice, the relative proportion remained similar. In contrast, in lesions from coinfected mice there was an increase in the infiltration of CD4 T cells and neutrophils but a loss in infiltrating inflammatory monocytes (Figure 4A-4B). The pus and ulcer formation observed in coinfected mice is consistent with excessive neutrophil recruitment and activation (Boaventura et al., 2010; Segel et al., 2011).

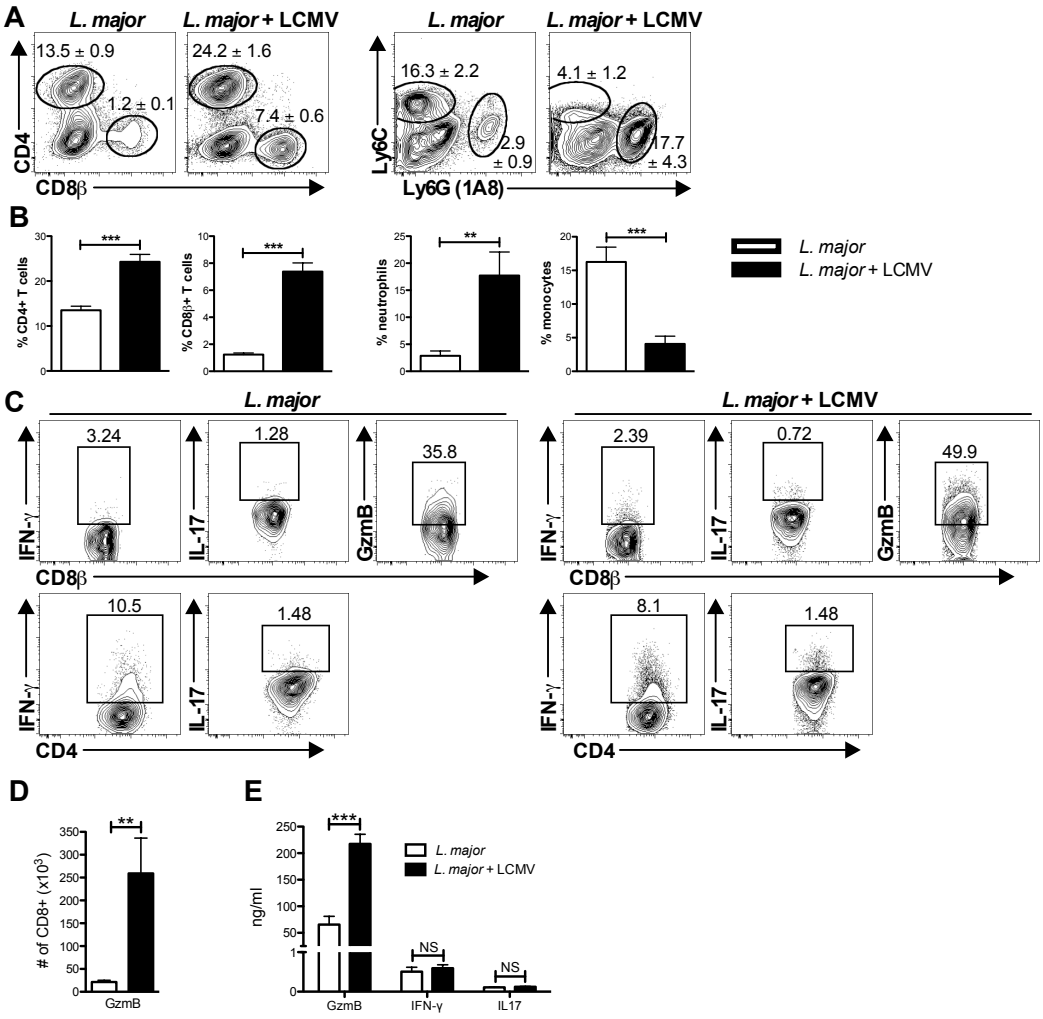

Figure 4 Coinfection with LCMV results in a significant increase in T cells and gzmB in the infected skin. Mice were infected with *L. major* or left uninfected for 2 weeks. Some mice from each group were then infected with LCMV. At the peak of lesion formation, 5 weeks after the initial infection with *L. major*, infected skin was harvested, digested, and stained with antibodies for T cells and myeloid cells (A). T cells were pregated on live, CD45+, CD11b- cells before the gates shown for CD4 and CD8 T cells. Myeloid cells were pregated on live, CD45+, CD11b+ cells before the gates shown for monocytes and neutrophils. Quantification of each cell population is shown as a percentage of total CD45+ cells (B). In addition to surface staining, cells from the skin were also incubated with BFA for 5 hours prior to intracellular staining for GzmB, IFN-γ, and IL-17 (C). Representative plots (C) and cell numbers for Gzm+ cells (D) are shown for CD4 and CD8 T cells. Whole ear tissue was homogenized and supernatants were anlzyed for GzmB, IFN-γ, and IL-17 by ELISA (E). Flow data are representative of two independent experiments (n=4-5 mice per group) and ear supernatant data is representative of a single experiment (n=4 mice per group). Percentages are shown as mean ± SEM. NS = not significant

We next wanted to determine if the functionality of the T cells present in the lesions could explain the increased pathology or recruitment of neutrophils. We isolated cells from the infected skin 5 weeks post *L. major* infection and incubated them with BFA but no additional stimulus in an effort to capture their functionality directly ex vivo. Consistent with previous work examining cytokine production by CD8 T cells from the infected skin there was little to no detectable IFN-γ or IL-17 production in either group (Figure 4C). In contrast, a significant proportion of CD8 T cells were expressing gzmB, with a higher percentage of CD8 T cells from coinfected mice expressing gzmB (Figure 4C). Similarly, while some IFN-γ and IL-17 production was detectable by CD4 T cells, there was no difference seen between the two groups (Figure 4C). When numbers of gzmB expressing CD8 T cells were calculated, there were overwhelmingly more gzmB expressing CD8 T cells in the coinfected mice than those infected with *L. major* alone (Figure 4D). Importantly, when infected skin was homogenized and the total proteins present were analyzed there was no difference in the amount of IFN-γ or IL-17 levels between the two groups, but coinfected mice had four times more gzmB than mice that had been infected with *L. major* alone (Figure 4E). Taken together these results indicate that IL-17 is not likely to be the factor responsible for increased neutrophil recruitment to the coinfected mice. Additionally, the increase in parasite burden does not appear to be due to a lack of IFN-γ production within the infected skin. The most abundant protein found in the skin was gzmB, potentially implicating cytotoxicity in the observed recruitment of neutrophils and resulting immunopathology.

We have shown previously that LCMV specific effector cells migrate into leishmanial lesions (Chapter 2). Additionally, it has been demonstrated that large numbers of LCMV specific CD8 T cells infiltrate a *Mycobacterium bovis* induced granuloma following an acute LCMV infection (Hogan et al., 2007). Based on these observations, our current

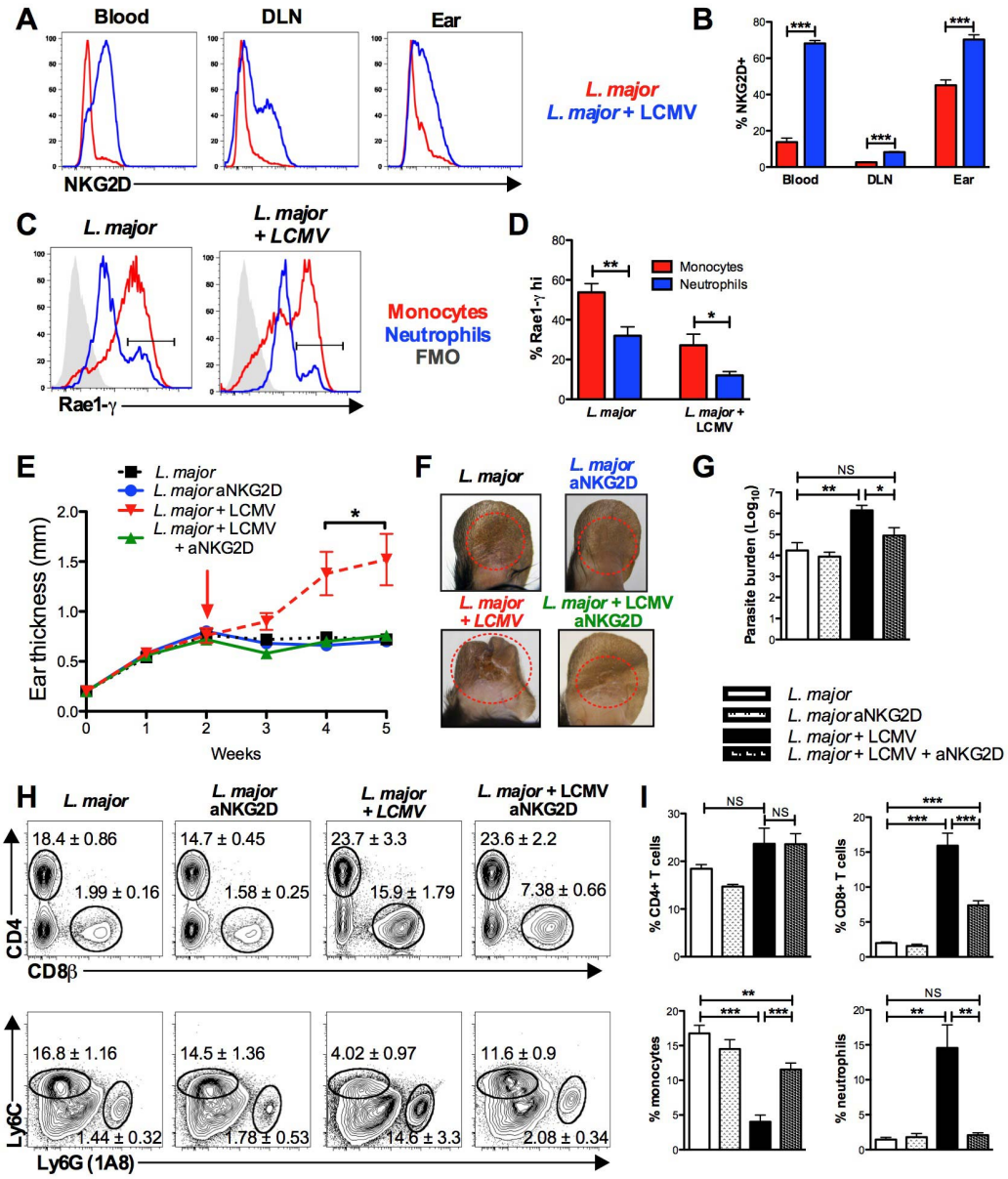

Figure 5. CD8 T cells induce immunopathology through engagement of NKG2D. Mice were infected with *L. major* or left uninfected for 2 weeks. Half of the mice in each group were then infected with LCMV and the peak of lesion formation (5 weeks post *L. major* infection) CD8 T cells from the blood, DLN, and infected skin were analzyed for surface expression of NKG2D (A-B). At 3 weeks post *L. major* infection, monocytes and neturophils in the lesions were analyzed for expression of Rae1-γ (C-D). Beginning on day 3 post infection with LCMV, some mice in each group were treated with NKG2D blocking antibody biweekly for the remainder of the experiment. Ear thickness was measured weekly (E). At the peak of lesion formation, 5 weeks after the initial infection with *L. major*, infected skin was photographed (F) before it was harvested and digested. Parasite burden was assessed using an LDA (G) and the remaining cells were stained with antibodies for T cells and myeloid cells (H). T cells were pregated on live, CD45+, CD11b- cells before the gates shown for CD4 and CD8 T cells (H, top). Myeloid cells were pregated on live, CD45+, CD11b+ cells before the gates shown for monocytes and neutrophils (H, bottom). Quantification of each cell population is shown as a percentage of total CD45+ cells (I). Data is representative of a single experiment (n=5 mice per group). Percentages are shown as mean ± SEM. NS = not significant

hypothesis is that LCMV infection activates and expands a large population of CD8 T cells that are recruited into the site of active inflammation in the leishmanial lesion. Future studies will be directed at testing this hypothesis.

Coinfection with LCMV results in high expression of NKG2D by CD8 T cells

Previous work has demonstrated that LCMV specific memory cells maintain expression of the NK cell receptor NKG2D (Chapter 3). This receptor has been demonstrated to act not only as a costimulatory receptor on CD8 T cells, but can also mediate direct, TCR-independent cytotoxicity (Groh et al., 2002; Jamieson et al., 2002; Meresse et al., 2004; Tietze et al., 2012; Verneris et al., 2004). We compared the expression of this receptor on CD44hi CD8 T cells in the blood, DLN, and infected skin in mice infected with *L. major* alone or coinfected with LCMV and *L. major* (Figure 5A). CD8 T cells from coinfected mice had significantly higher expression of NKG2D in all three tissues (Figure 5B).

Monocytes in *L. major* infected skin express higher levels of Rae1-γ than neutrophils

We have previously shown that one of the ligands for NKG2D, Rae1-γ, is highly expressed in the skin following *L. major* infection (Chapter 3). We wanted to further demonstrate the relative expression of this ligand by different cells types in the lesion. Examining the expression of Rae1-γ by monocytes and neutrophils we found that both cell types expressed Rae1-γ. However, there appeared to be two levels of expression of this ligand by monocytes and neutrophils, a population of high expressers and a population of low expressers (Figure 5C). In both mice infected with *L. major* alone and coinfected mice, monocytes expressed higher levels of Rae1-γ than neutrophils, with the majority of monocytes falling in the high expressing population (Figure 5D). It is

currently unknown what distinguishes these two populations of high and low expression of Rae1-γ in monocytes and neutrophils. We might predict that cells infected with parasite will express higher levels, and use of fluorescent parasites will allow us to determine if this is the case.

Immunopathology in coinfected mice is mediated by engagement of NKG2D

Coinfection of *L. major* infected mice with LCMV resulted in the infiltration of NKG2D and gzmB expressing CD8 T cells. Knowing that NKG2D expressing memory CD8 T cells mediated increased immunopathology through this receptor (Chapter 3), we wanted to test whether the observed immunopathology during coinfection was also dependent on engagement of this receptor. We followed the same infection time line as before, adding treatment with NKG2D blocking antibodies biweekly beginning 3 days after LCMV infection. Treatment of coinfected mice with anti-NKG2D completely blocked the increase in ear thickness (Figure 5E). The lesions in these mice were visibly smaller and had no ulceration (Figure 5F). Strikingly, this treatment not only blocked the immunopathology, but it also restored control of parasite burden (Figure 5G). Coinfected mice treated with anti-NKG2D had no change in the recruitment of CD4 T cell to the lesion (Figure 5H and 5I). Interestingly, while there were still significantly more CD8 T cells in anti-NKG2D treated coinfected mice than mice that had been infected with *L. major* alone, there were approximately half as many as in control coinfected mice (Figure 5H and 5I). Anti-NKG2D treatment also led to a partial restoration in monocyte infiltration and completely blocked the excessive infiltration of neutrophils in the coinfected mice (Figure 5H and 5I). Currently, the data demonstrates that the NKG2D pathway is responsible for the exacerbated lesion formation and increased parasite

burden, and that blockade of this pathway completely blocks the pathology and restores parasite control.

4.5 Discussion

This study explores the impact of LCMV infection on the progression of an established leishmanial lesion. Unexpectedly, this coinfection results in not only significantly exacerbated disease severity but also transiently impaired parasite control. Given the chronic nature of *Leishmania* infection, understanding the impact of additional infections during the course of active disease has critical applications for human health. Much study in this area has focused on the impact of coinfections that would be predicted to induce an alternate type II immune response, like helminths and other intestinal parasites. A mouse model of *Schistosoma mansoni* and *L. major* coinfection resulted in a delayed IFN-γ and increased IL-4 response to *L. major*, ultimately leading to increased parasite burden and delayed healing (La Flamme et al., 2002). A clinical study of patients in Brazil found that patients who were coinfected with an intestinal helminth at the time of *Leishmania* diagnosis took longer to heal and exhibited increase type II immune responses as measured by serum IgE levels (O'Neal et al., 2007). The opposite effect has also been shown using strong type I inducing infections like *T. gondii* and *Listeria* coinfections with *Leishmania* to protect otherwise susceptible strains of mice (Santiago et al., 1999; Tabbara et al., 2004). Here we see not only increased pathology, but also an increase in the peak parasite burden of coinfected mice. Rather than an increased IFN-γ response in the coinfected mice that we would have predicted based on previous work, there was a defect in *Leishmania* specific IFN-γ production observed at 3 weeks post *L. major* (Figure 2A-2D). LCMV induces an extensive T cell response that can account for at least 80% and possibly as much at 95% of the CD8 T cell pool at its peak (Figure 3B and Masopust et al., 2007; Murali-Krishna et al., 1998). This expansion

also leads to a shift in the CD4:CD8 T cell ratio in the DLN and may decrease the number of leishmania responsive cells present there enough to result in the IFN-γ production defect that we see. Following this massive expansion, there can be also be a period of time during which cells from these mice are refractory to further stimulation, possible resulting in the lack of anti-leishmanial IFN-γ production (Butz and Southern, 1994; Saron et al., 1990). Other evidence indicates that the high levels of Type I IFNs present during an acute viral infection like LCMV can also result in a decreased responsiveness by T cells following infection (Marshall et al., 2011; Redford et al., 2014; Wilson et al., 2013). This blunted anti-leishmanial immune response was seen in both CD4 and CD8 T cells and may be partially responsible for the increased parasite burden seen at this time point.

This data also highlights the fine balance between a potentially protective increase in the immune response and the generation of immunopathology. There is increasing evidence that CD8 T cells, despite their ability to produce IFN-γ, can also act to promote tissue damage (Chapter 2 and 3 and Novais et al., 2013; Santos et al., 2013). Patient lesions have been shown to contain large numbers of CD8 T cells, many of which appear to be specific for pathogens other than *Leishmania* (Da-Cruz et al., 2010). This observation was unexpected, given that many of the CD8 observed in both mouse and patient lesions expressed gzmB, an effector protein usually associated with antigen specific responses (Dantas et al., 2013; Faria et al., 2009). These studies identify NKG2D as a potential mechanism by which gzmB expressing non-antigen specific CD8 T cells could be inducing pathology and potentially parasite spread. In vitro experiments indicate that killing of a *Leishmania* infected cell by CD8 T cells does not kill the parasite, but instead releases infectious parasites to potentially infect surrounding cells (Smith et al., 1991). As a result, excessive lysis of infected cells within the lesion by LCMV

derived effector CD8 T cells through NKG2D could not only explain the extensive tissue damage, but also the increase in parasite burden at the peak of infection.

Treatment with antibodies that blocked NKG2D engagement resulted in decreased recruitment of CD8 T cells to the infected skin. There are several possible reasons that NKG2D treatment might have this effect. NKG2D engagement with its ligand has been shown to be sufficient for recruitment and retention of CD8 T cells into the pancreas (Markiewicz et al., 2012). Blockade of this interaction may lead to a decreased retention of effector CD8 T cells in lesion. NKG2D can also act as a costimulatory molecule and it is not known what role this pathway plays in during the priming of CD8 T cells during LCMV infection. A recent paper even suggests that blockade of NKG2D during acute LCMV infection actually enhances the CD8 T cell response (Lang et al., 2012). At 5 weeks post *L. major* infection, the percentage of both total and activated CD8 T cells in the blood of coinfected or anti-NKG2D treated coinfected mice was the same, suggesting a comparable immune response in both groups (data not shown). Additionally, the DLNs of these mice were the same size and cellularity (data not shown). The impact of NKG2D blockade on the LCMV specific immune response and viral control will be examined in future work.

At 5 weeks post *L. major* infection there was a notable loss of the monocyte population in coinfected mice that was not observed when LCMV immune mice were infected with *L. major* (Figure 4A and Chapter 3). However, at 3 weeks and 7 weeks post infection, monocyte populations return to comparable numbers as mice infected with *L. major* alone (data not shown). Monocytes in the infected skin express high levels of the NKG2D ligand Rae1-γ whereas neutrophils express much lower levels of this protein (Figure 5C and 5D). A possible explanation for the loss at the peak of infection is

targeted killing of the highly expressing Rae1-γ monocyte population by activated CD8 effector cells. Neutrophils have been shown to effectively take up parasites, but are less efficient at killing them than monocytes, instead providing a potential 'home' for parasite replication, consistent with the increased parasite burden observed in coinfected mice (Laskay et al., 2003). Alternatively, LCMV effector CD8 T cells may produce different chemokines or cytokines than LCMV memory CD8 T cells upon entering the inflammatory environment of the leishmanial lesion. Memory CD8 T cells produce CCL3 and/or CCL4 in response to secondary infection with LCMV or Listeria (Narni-Mancinelli et al., 2007; Schenkel et al., 2013). However, no work has been done to determine what chemokines are produced by CD8 T cells upon entry into a non-antigenic inflamed environment or whether effector and memory CD8 T cells will respond differently to this environment. Production of excessive neutrophil specific chemokine like CCL4 could result in the preferential recruitment of these cells to the infected skin (Kobayashi, 2008). Understanding the regulation of CD8 T cell effector functions like chemokine and cytokine production in multi-infection settings and complex inflammatory environments is an important question to address as we seek to better understand immune responses in patients.

Chronic and persistent infections represent a significant disease burden worldwide. *Leishmania* infection alone impacts people in 98 countries, with 1-2 million newly infected individuals each year (Alvar et al., 2012). In addition, *Leishmania* is endemic to areas of the world where intestinal helminth and HIV infections are also highly prevalent. The incidence of *Leishmania*/HIV coinfection is growing rapidly and is associated with an increased rate of leishmanial recurrence and a decreased responsiveness to therapy (Couppie et al., 2004). A study of cutaneous *Leishmania* patients in Brazil at the time of diagnosis revealed that 88.3% of the patients were coinfected with at least 1 species of

helminth, with 73% of the patients harboring multiple species (O'Neal et al., 2007). This cohort was followed and found that coinfected individuals took longer to heal their lesions. Our work demonstrates that the coinfection does not have to be with another chronic pathogen; rather leishmanial disease progression can be affected by an acute viral infection. Overall, this work highlights the need for a more systemic understanding of bystander immune responses that are occurring during chronic infections in order to improve therapies and create more targeted and effective treatments.

Chapter 5 Discussion

The focus of this work has been on understanding the role of CD8 T cells, particularly during heterologous infections, on the disease course of *L. major* infection. The findings have demonstrated that CD8 T cells in the skin appear to have a cytotoxic phenotype, which is consistent with other work from our lab and observations of CD8 T cells in human lesions (Dantas et al., 2013; Faria et al., 2009; Novais et al., 2013; Santos et al., 2013). Using unrelated, acute infections to generate a large population of memory CD8 T cells, we demonstrated that bystander memory cells can be recruited into the lesion and exacerbate the course of disease (Figure 1). This immunopathological property was not limited to memory cells, as coinfection of mice with LCMV after the formation of a lesion also exacerbated disease. In both cases, the pathology was dependent on the engagement of NKG2D with its ligands, resulting in cell death, tissue damage, and the recruitment of inflammatory cells that perpetuated the cycle of inflammation (Figure 1).

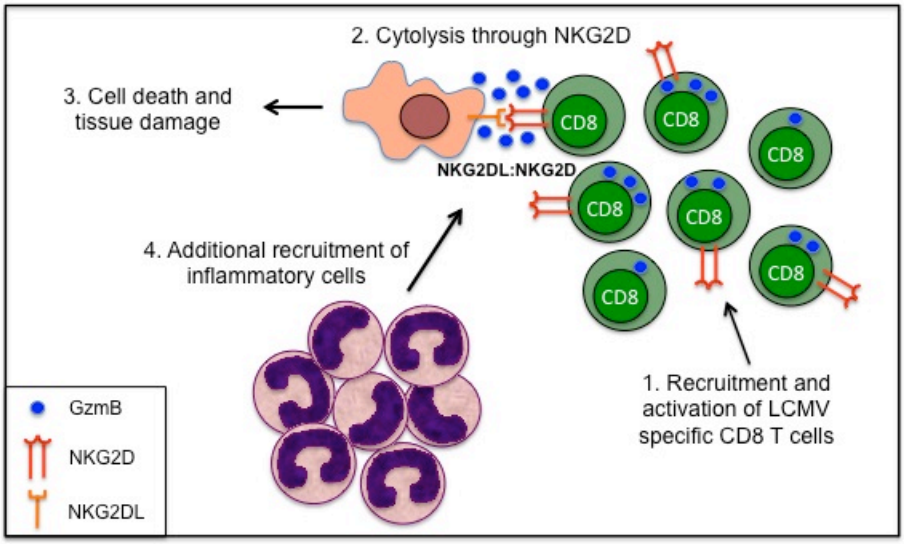

Figure 1. Induction of pathology by bystander CD8 T cells through engagement of NKG2D. Bystander CD8 T cells expressing NKG2D are recruited to the site of *L. major* infection and express gzmB upon entering the inflammatory environment. Activated CD8 T cells encounter high expression of NKG2D ligands, like Rae1-γ, by cells in the infected skin and lyse these cells. The excessive cell death leads to tissue damage and the recruitment of additional inflammatory cells that themselves can upregulate NKG2D ligands, perpetuating the cycle of cell death and inflammation.

Overall, these data raise several important questions. Here we will discuss the implications for this work as we think about the use of a mouse model to study infectious diseases, the innate-like functioning of bystander memory cells and its impact on future infections, the skin as a specific immunologic niche, and how these findings might impact the treatment of leishmaniasis.

Innate functions of CD8 T cells: bystander activation and its implications for human health

The role of CD8 T cells in both primary and secondary adaptive immune responses has been well characterized. Intricate positive and negative selection ensures that these T cells are able to respond only to appropriate antigens and complex regulatory networks exist to ensure non-responsiveness to self (Starr et al., 2003). The formation of long lasting, pathogen specific immunologic memory is one of the hallmarks of adaptive immunity. However, more recently the field has begun to appreciate an innate-like functioning of CD8 T cells that is independent of antigen specific signals. While naïve T cells require signals through their TCR, previously activated effector or memory cells can upregulate substantial effector functions in response to cytokine signals in their environment. This is referred to as bystander activation and has been historically somewhat controversial. Initially, the massive expansion of T cells following viral infection was thought to be due to bystander activation, but the advent of tetramer technology has demonstrated that antigen specific responses can be much larger than was previously thought (Masopust et al., 2007; Murali-Krishna et al., 1998). Use of transgenic cells has further demonstrated the capacity for antigen specific proliferation, but has also made it clear that memory cells can be activated independent of antigen specificity by a subsequent, unrelated pathogen (Berg et al., 2003). This phenomenon has been termed heterologous immunity and has been demonstrated between different

strains of closely related viruses, different members of the same virus group, and also among completely unrelated pathogens like parasites, bacteria and viruses (Reviewed in Sharma and Thomas, 2014). In many situations heterologous immunity can result in increased protection from subsequent infections (Berg et al., 2003; Mathurin et al., 2009; Soudja et al., 2012), while in other cases, stimulation of heterologous immunity results in increased immunopathology or the induction of autoimmune diseases (Chen et al., 2001; Meresse et al., 2004). Understanding the balance between protective and pathologic effector functions of bystander cells has important implications for human health.

The first effector function of bystander CD8 T cells to be implicated in the induction of both protection and immunopathology is cytokine production. When considering the innate functioning of bystander cells, production of cytokines as a means of influencing future infections is logical. We know that memory cells can rapidly produce cytokines in response to inflammatory signals (Freeman et al., 2012). Cytokines can influence the cells around them, activating or skewing the type of immune response being mounted, irrespective of antigen specificity. In some settings, cytokines can result in control of pathogens, while in others, cytokines can cause tissue damage and inflammation (Angulo and Fresno, 2002; Wack et al., 2011). This can vary depending on the specific cytokine, the pathogen involved, the local concentration of cytokine, and the tissue where the response is taking place. While excessive cytokine production by bystander cells could certainly be an issue, many innate cells produce cytokines in a similarly 'non-specific' way without catastrophic consequences. However, there is increasing evidence that bystander CD8 T cells are not just functioning as cytokine producers, but can also have cytolytic activity. The notion that a bystander CD8 T cell can not only secrete cytokines, but can kill independently of its TCR is much more teleologically alarming.

Historically, when bystander killing was observed, it was explained as a byproduct of cross-reactivity or potentially the expression of two TCRs with differing specificities on the same cell (Nahill and Welsh, 1993; Selin et al., 2006; Sheil et al., 1987). But the use of tetramers and transgenic technology has allowed the convincing demonstration of bona fide bystander killing that is independent of antigen specificity (Chu et al., 2013; Meresse et al., 2004). One study demonstrated that Sendai virus specific bystander cells expressed high levels of gzmB in the lung following Influenza infection and led to increased control of Influenza virus early during the infection (Kohlmeier et al., 2010). Surprisingly, they went on to show that these cells lysed Sendai pulsed target cells, despite the complete absence of Sendai virus from the lungs at the time of Influenza infection, but failed to examine whether they could lyse Influenza pulsed target cells. They maintained that the increased cytotoxicity was antigen specific but never explained how Sendai specific cytotoxic potential was controlling Influenza infection. More recent work, including the work shown here, has provided a much more satisfying explanation for how bystander cells expressing cytotoxic proteins could be influencing unrelated infections, through the engagement of NKG2D.

Memory CD8 T cells have been shown to express the NK cell receptor NKG2D, and binding of this receptor can lead to direct, TCR-independent cytotoxicity. Our work highlights a specific instance in which bystander CD8 T cells induce cytolysis through engagement of NKG2D. Blockade of this interaction completely blocked the immunopathology caused by CD8 T cells, but targeting of this pathway for potential therapies will require a much greater understanding of how and when signaling through this pathway occurs in CD8 T cells. Self-reactive NKG2D expressing CD8 T cells have also been shown to play a role during several autoimmune diseases, including Celiac disease and diabetes, through MHC unrestricted cytotoxicity (Meresse et al., 2004;

Ogasawara et al., 2004). The demonstration of NK-cell-like killing by CD8 T cells, particularly during autoimmune disease, raises some important questions about how this process is regulated. NK cells express a complex network of inhibitory and activating receptors that constantly signal, the sum of which determines whether the NK cell will 'act' or not (Pegram et al., 2011). While some work has been done to examine the expression of these NK cell receptors on CD8 T cells, much less is known about how they impact the functionality of CD8 T cells and how they interact with each other to provide positive and negative feedback. Conventionally, NKG2D has been reported to act as a costimulatory molecule during the priming of CD8 T cells, but even this finding seems to be very context and infection dependent. It will be important to understand if signaling through these receptors is different not only during various infections, but also how signaling varies in naïve versus effector versus memory T cells, as we already know that downstream TCR signaling is distinct depending on the activation state of the cell (Kersh et al., 2003). In all, our understanding of NK cell receptor expression, interaction, regulation, and function on CD8 T cells is lacking and represents an important potential component of overall CD8 T cell function.

Some of the first evidence that CD8 T cells expressed NKG2D and could kill non-specifically through this receptor was in the setting of cancer immunotherapy. Treatment of T cells with high doses of IL-2 induces potent cytotoxic effector cells that express both gzmB and NKG2D (Tietze et al., 2012; Verneris et al., 2004). We also saw the induction of gzmB by CD8 T cells in response to IL-2 in vitro, although its effect on NKG2D expression was not examined in our experiments (Chapter 1, Figure 5). Significant anti-tumor cytotoxicity in response to IL-2 treatment was seen even when using transgenic cells against non-antigen expressing tumors and could be blocked by treatment with anti-NKG2D. This data demonstrates a potential opportunity to augment anti-tumor

responses by targeting this pathway more specifically. As chimeric antigen receptors become more complex with the addition of costimulatory domains, NKG2D stands out as a prospect. This data also suggests that immunotherapies utilizing high dose IL-2 or the expansion ex vivo of T cells in IL-2 or IL-15 could result in unwanted bystander activation, killing, and potentially the induction of autoimmunity.

Overall, innate functioning of both antigen specific and bystander memory cells occurs in vivo. Our current understanding of how these functions are induced and regulated is lacking. Expression of innate receptors like NKG2D represents an exciting new avenue into understanding the complexities of T cell responses that occur in patients. The data suggest that finding ways to both inhibit and augment signaling through this pathway warrants further, careful study.

Studying T cells in a mouse

The textbook description of T cell activation mandates engagement of the TCR to 'license' a T cell to respond in an antigen specific manner. Following an initial expansion and contraction phase, a stable memory population of T cells is formed and activated upon future exposure to the initiating pathogen. Unfortunately, this is where our understanding of T cell responses, gleaned mostly from mouse studies, begins to break down. We have long appreciated that effector and memory T cells can respond to their environment both in conjunction with and independently of TCR signals, but the true breadth of this response in vivo is only partially understood. The complexity of the different microenvironments in which T cells must respond include duration, receptor expression, nutrient and oxygen availability, the structure of the reticular network, and location- both across different tissues and at different sites within a tissue- to name a few. Add to this the spectrum of activation states (naïve versus effector versus memory)

of the T cells involved and understanding or predicting how a T cell population will respond to a given infection becomes difficult, if not impossible. This unmanageable complexity is what makes the specific pathogen free (SPF), genetically identical mouse model so attractive. However, this decrease in complexity comes at a cost, and as the field moves forward it may ultimately limit the possible applications for understanding human health and disease.

Our studies have identified and attempted to address at least one of the issues with the SPF mouse model: using immunologically 'naïve' mice to study the immune response to pathogens. While it is no surprise that there does not exist an immunologically naïve human, we base our understanding of immune responses on data collected almost exclusively from animals in this naïve state. It is becoming increasingly clear that previous immunological exposure has a significant impact on the character and quality of the immune response that is mounted. The studies described here outline the consequences of two different scenarios, both of which can be expected to occur during the natural course of infection with *Leishmania*. The first is an example of the impact of immunological history on future immune responses, where a previously cleared, unrelated acute viral or bacterial infection altered the magnitude and quality of the immune response to *Leishmania*. Here parasite burden was not impacted, but significant immunopathology was induced. This result is reminiscent of the clinical spectrum of disease that is seen in patients and allows one to speculate that individual immunologic patient histories may be part of the story when predicting disease severity and response to treatment. The second scenario is a direct coinfection, where mice are infected with a virus while a lesion is present in the skin. Again we see dramatically altered immunopathology and a surprising impact on both parasite burdens and the anti-leishmanial immune response.

Previous infection induces several important changes to the immune system that can impact future responses. One of these changes that is particularly important when studying immune responses in the skin, is the seeding of immune cells into non-lymphoid tissues. Following priming of an adaptive immune response in the spleen, cells acquire promiscuous homing capabilities and are dispersed throughout the body, presumably to ensure complete clearance of the pathogen (Liu et al., 2006; Masopust et al., 2004; Masopust et al., 2010; Stevceva et al., 2002). There is a growing appreciation that these cells become resident within peripheral tissues and can remain there indefinitely (Ariotti et al., 2012; Gebhardt et al., 2009; Mackay et al., 2013). As a result, over time human peripheral tissues acquire resident memory T cells specific for numerous pathogens. The skin is a particularly striking example when comparing the SPF mouse model to data from humans. Quantification of T cells in human skin samples revealed that nearly twice as many cells are present within the skin at steady state as are present in the circulation (Clark et al., 2006). This is in stark contrast to the number of T cells observed in the ear skin from SPF mice, where there is a small population of CD4 T cells and CD8 T cells are virtually absent (Chapter 2, Figure 1). Recent work has demonstrated the dramatic difference that having CD8 T cells present within the female reproductive tract can have on the initiation speed and magnitude of a protective immune response to a challenge at this site (Schenkel et al., 2013). Alternatively, our work has highlighted the detrimental role these cells can play in subsequent immune response through the induction of immunopathology. While the details are likely case specific, the conclusion remains the same: previous exposure to pathogens changes tissues and impacts future immune responses. Despite the convenience of the mouse model, this is a complication that cannot be ignored if we are

to further our understanding of the immune response as a system that is more than a sum of its individual parts.

Another problematic aspect of studying immune response in the SPF mouse model is the use of genetically identical, inbred mouse strains. Leishmaniasis provides a perfect opportunity to make this point, as different genetic strains exhibit wildly different abilities to control parasite replication. Often the strain differences seen during *Leishmania* infection are over simplified into the observation that protected strains (BL6) having an inherently more TH1 biased response, whereas as susceptible strains (BALB/c) are skewed towards a TH2 response. While this observation has provided us with important information about the requirements to control *Leishmania* infection, it complicates our translation of these studies to patients. It is easy to fall into the trap of thinking of patients as falling somewhere on the spectrum between a BL6 and BALB/c mouse, when in reality we may have artificially chosen two genetic representatives whose homologues rarely, if ever, appear in an actual human population. It has been shown that MHC diversity significantly impacts an individual's ability to control certain pathogens, and there are many other allelic differences that are more difficult to track at a population level that likely have a significant impact on the immune response as well (Penn et al., 2002). Assessing genetic variation in human patients as it relates to disease severity is increasingly common and represents an exciting and informative step in the study of human disease. Again, the usefulness of the mouse model is undeniable, but ultimately it is crucial to keep in mind the limitations when conducting translational research.

Leishmaniasis: CD8 T cells, therapy and the skin

Skin infections have several unique characteristics that make them both interesting and challenging. As a barrier surface, the skin represents a unique niche for commensals, pathogens, and immune cells. Like the gut, a delicate balance of regulation and activation must be achieved to maintain homeostasis while still protecting against pathogens. The microbiome and regulatory components of the skin immune system cannot be ignored when considering immune responses occurring at this site. Cutaneous commensal bacteria drive both protective IFN-γ production and pathologic lesion formation during *L. major* infection (Naik et al., 2012). It is unclear what role commensal bacteria may play in NKG2D mediated killing by CD8 T cells, but altering the gut commensal flora had a significant impact on the expression of NKG2D ligands (Hansen et al., 2013). In this study, treatment with vancomycin led to decreased ligand expression by the intestinal epithelium, whereas treatment with ampicillin led to a dramatic increase in the expression of NKG2D ligands. This opens the possibility that there may also be a link between commensals and the expression of NKG2D ligands in the skin and that expression could be altered by topical antibiotic treatment during *Leishmania* infection.

Regulatory T cells are present at a particularly high proportion within the skin and are required for the maintenance of homeostasis at this site (Dudda et al., 2008). They also play an important role in regulating the persistence of *Leishmania* parasite in the skin after overt disease has healed (Belkaid et al., 2002). Neutralization of IL-10 led to sterile cure of parasite but a concomitant loss of immunity to reinfection, emphasizing the importance of regulation of the immune response during *Leishmania* infection. Blockade of IL-10 receptor during *L. major* infection also resulted in increased IFN-γ production by CD8 T cells in skin, which may contribute to the sterile cure observed (unpublished

data). This suggests that regulation of CD8 function is occurring in the skin, potentially by IL-10, that inhibits IFN-γ production by CD8 T cells and may play a role in causing them to adopt a cytotoxic phenotype. Here we have shown that IL-15 is in part responsible for the expression of gzmB by CD8 T cells in the skin. It is likely that the cytotoxic phenotype observed by both antigen specific and non-specific cells in the skin is the cumulative result of inflammatory signals that enforce the expression of gzmB and inhibitory signals, like IL-10, that repress cytokine production. Moving forward, it will be important to establish a better understanding of the specific inflammatory and regulatory signals present in the skin during *Leishmania* infection, with the hopes of being able to tip the balance in favor of protection rather pathology.

When considering new therapies to target these pathologic CD8 T cells, we have identified NKG2D as a potential new pathway. While we treated the mice systemically with NKG2D blocking antibodies, another recent study employed a localized, intranasal treatment during influenza infection and was able to block CD8 cytotoxicity (Sckisel et al., 2014). The cutaneous nature of *Leishmania* infection raises the possibility of a localized treatment with blocking reagents that could significantly minimize off target effects. However, blocking antibody is not the only option for negatively regulating this pathway. Several groups have discovered soluble forms of NKG2D ligands in the sera of cancer patients (Champsaur and Lanier, 2010). Presence of these ligands in the sera of melanoma patients was a strong indicator of a poor prognosis (Paschen et al., 2009). Incubation of CD8 T cells with this sera or purified soluble ligands significantly decreased the expression levels of NKG2D on these cells and impaired their antitumor activity (Groh et al., 2002). While in the context of cancer, the repressive effects of soluble NKG2D ligands are detrimental, in the setting of *Leishmania* infection it

represents a potential therapeutic agent to minimize NKG2D/CD8 mediated immunopathology.

Overall, this work demonstrates the pathological function of bystander CD8 T cells during *Leishmania* infection and ultimately the impact that previous infections can have on disease severity. It suggests that the spectrum of disease seen in patients may be due in part to their individual immunological history. While we cannot, and would not want, to eliminate the memory cells that are inducing pathology, here we show a very specific way to inhibit their activity in the lesions. NKG2D blockade represents an immune modulator that allows for the repression of pathologic cytotoxicity without inhibition of protective immune responses. Treatment with antibodies against NKG2D was effective in mice, but soluble NKG2D ligands may be an even simpler solution in patients that has broader applications for not only leishmaniasis but also many autoimmune diseases.

BIBLIOGRAPHY

Alvar, J., Aparicio, P., Aseffa, A., Den Boer, M., Canavate, C., Dedet, J., Gradoni, L., Ter Horst, R., Lopez-Velez, R., and Moreno, J. (2008). The relationship between leishmaniasis and AIDS: the second 10 years. Clin. Microbiol. Rev. 21, 334-+.

Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., and WHO Leishmaniasis Control Team. (2012). Leishmaniasis Worldwide and Global Estimates of Its Incidence. Plos One 7, e35671.

Andreani, G., Lodge, R., Richard, D., and Tremblay, M.J. (2012). Mechanisms of interaction between protozoan parasites and HIV. Curr. Opin. HIV AIDS 7, 276-282.

Angulo, I., and Fresno, M. (2002). Cytokines in the Pathogenesis of and Protection against Malaria. Clinical and Diagnostic Laboratory Immunology 9, 1145-1152.

Antoniou, M., Gramiccia, M., Molina, R., Dvorak, V., and Volf, P. (2013). The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the Mediterranean region. Eurosurveillance 18, 54-61.

Ariotti, S., Beltman, J.B., Chodaczek, G., Hoekstra, M.E., van Beek, A.E., Gomez-Eerland, R., Ritsma, L., van Rheenen, J., Maree, A.F.M., Zal, T.*, et al.* (2012). Tissue-resident memory CD8(+) T cells continuously patrol skin epithelia to quickly recognize local antigen. Proc. Natl. Acad. Sci. U.S.A. 109, 19739-19744.

Bacellar, O., Faria, D., Nascimento, M., Cardoso, T.M., Gollob, K.J., Dutra, W.O., Scott, P., and Carvalho, E.M. (2009). Interleukin 17 Production among Patients with American Cutaneous Leishmaniasis. J. Infect. Dis. 200, 75-78.

Badovinac, V.P., Haring, J.S., and Harty, J.T. (2007). Initial T Cell Receptor Transgenic Cell Precursor Frequency Dictates Critical Aspects of the CD8+ T Cell Response to Infection. Immunity 26, 827-841.

Beadling, C., and Slifka, M.K. (2004). How do viral infections predispose patients to bacterial infections? Curr. Opin. Infect. Dis. 17, 185-191.

Beadling, C., and Slifka, M.K. (2005). Differential regulation of virus-specific T-cell effector functions following activation by peptide or innate cytokines. Blood 105, 1179-1186.

Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., and Sacks, D. (2000). A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J. Immunol. 165, 969-977.

Belkaid, Y., Piccirillo, C., Mendez, S., Shevach, E., and Sacks, D. (2002). CD4(+)CD25(+) regulatory T cells control Leishmania major persistence and immunity. Nature 420, 502-507.

Belkaid, Y., Von Stebut, E., Mendez, S., Lira, R., Caler, E., Bertholet, S., Udey, M.C., and Sacks, D. (2002). CD8+ T Cells Are Required for Primary Immunity in C57BL/6 Mice Following Low-Dose, Intradermal Challenge with Leishmania major. J. Immunol. 168, 3992-4000.

Berg, R.E., Crossley, E., Murray, S., and Forman, J. (2003). Memory CD8+ T Cells Provide Innate Immune Protection against Listeria monocytogenes in the Absence of Cognate Antigen. J. Exp. Med. 198, 1583-1593.

Berg, R.E., and Forman, J. (2006). The role of CD8 T cells in innate immunity and in antigen non-specific protection. Curr. Opin. Immunol. 18, 338-343.

Berg, R., Cordes, C., and Forman, J. (2002). Contribution of CD8(+) T cells to innate immunity: IFN-gamma secretion induced by IL-12 and IL-18. Eur. J. Immunol. 32, 2807-2816.

Boaventura, V.S., Santos, C.S., Cardoso, C.R., de Andrade, J., Dos Santos, W.L.C., Clarêncio, J., Silva, J.S., Borges, V.M., Barral-Netto, M., Brodskyn, C.I., and Barral, A. (2010). Human mucosal leishmaniasis: Neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. Eur. J. Immunol. 40, 2830-2836.

Boon, A.C.M., de Mutsert, G., van Baarle, D., Smith, D.J., Lapedes, A.S., Fouchier, R.A.M., Sintnicolaas, K., Osterhaus, A.D.M.E., and Rimmelzwaan, G.F. (2004). Recognition of homo- and heterosubtypic variants of influenza A viruses by human CD8(+) T lymphocytes. J. Immunol. 172, 2453-2460.

Bou Ghanem, E.N., Nelson, C.C., and D'Orazio, S.E.F. (2011). T Cell-Intrinsic Factors Contribute to the Differential Ability of CD8+ T Cells To Rapidly Secrete IFN-γ in the Absence of Antigen. J. Immunol. 186(3):1703-12.

Bousoffara, T., Louzir, H., Ben Salah, A., and Dellagi, K. (2004). Analysis of granzyme B activity as a surrogate marker of Leishmania-specific cell-mediated cytotoxicity in zoonotic cutaneous leishmaniasis. J. Infect. Dis. 189, 1265-1273.

Brehm, M.A., Pinto, A.K., Daniels, K.A., Schneck, J.P., Welsh, R.M., and Selin, L.K. (2002). T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. Nat. Immunol. 3, 627-634.

Brodskyn, C.I., Barral, A., Boaventura, V., Carvalho, E., and Barral-Netto, M. (1997). Parasite-driven in vitro human lymphocyte cytotoxicity against autologous infected macrophages from mucosal leishmaniasis. J. Immunol. 159, 4467-4473.

Brojorge.K, and Volkert, M. (1974). Defects in Immune System of Mice Infected with Lymphocytic Choriomeningitis Virus. Infect. Immun. 9, 605-614.

Butcher, E.C., and Picker, L.J. (1996). Lymphocyte Homing and Homeostasis. Science 272, 60-67.

Butz, E., and Southern, P. (1994). Lymphocytic Choriomeningitis Virus-Induced Immune Dysfunction - Induction of and Recovery from T-Cell Anergy in Acutely Infected Mice. J. Virol. 68, 8477-8480.

Butz, E.A., and Bevan, M.J. (1998). Massive Expansion of Antigen-Specific CD8+ T Cells during an Acute Virus Infection. Immunity 8, 167-175.

Carayannopoulos, L., Naidenko, O., Fremont, D., and Yokoyama, W. (2002). Cutting edge: Murine UL16-binding protein-like transcript 1: A newly described transcript encoding a high-affinity ligand for marine NKG2D. J. Immunol. 169, 4079-4083.

Centers for Disease Control and Prevention (CDC). (2010). Estimates of deaths associated with seasonal influenza --- United States, 1976-2007. MMWR.Morbidity and mortality weekly report 59, 1057-1062.

Cerwenka, A., Baron, J., and Lanier, L. (2001). Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. Proc. Natl. Acad. Sci. U. S. A. 98, 11521-11526.

Champsaur, M., and Lanier, L.L. (2010). Effect of NKG2D ligand expression on host immune responses. Immunol. Rev. 235, 267-285.

Chen, A.T., Cornberg, M., Gras, S., Guillonneau, C., Rossjohn, J., Trees, A., Emonet, S., de la Torre, J.C., Welsh, R.M., and Selin, L.K. (2012). Loss of Anti-Viral Immunity by Infection with a Virus Encoding a Cross-Reactive Pathogenic Epitope. Plos Pathogens 8, e1002633.

Chen, H.D., Fraire, A.E., Joris, I., Brehm, M.A., Welsh, R.M., and Selin, L.K. (2001). Memory CD8(+)T cells in heterologous antiviral immunity and immunopathology in the lung. Nat. Immunol. 2, 1067-1076.

Chen, H.D., Fraire, A.E., Joris, I., Welsh, R.M., and Selin, L.K. (2003). Specific history of heterologous virus infections determines anti-viral immunity and immunopathology in the lung. Am. J. Pathol. 163, 1341-1355.

Chu, T., Tyznik, A., Roepke, S., Berkley, A., Woodward-Davis, A., Pattacini, L., Bevan, M., Zehn, D., and Prlic, M. (2013). Bystander-Activated Memory CD8 T Cells Control Early Pathogen Load in an Innate-like, NKG2D-Dependent Manner. Cell Rep. 3, 701-708.

Clark, R.A., Chong, B., Mirchandani, N., Brinster, N.K., Yamanaka, K., Dowgiert, R.K., and Kupper, T.S. (2006). The vast majority of CLA(+) T cells are resident in normal skin. J. Immunol. 176, 4431-4439.

Colmenares, M., Kima, P., Samoff, E., Soong, L., and McMahon-Pratt, D. (2003). Perforin and gamma interferon are critical CD8(+) T-cell-mediated responses in vaccine-induced immunity against Leishmania amazonensis infection. Infect. Immun. 71, 3172-3182.

Conceicao-Silva, F., Hahne, M., Schroter, M., Louis, J., and Tschopp, J. (1998). The resolution of lesions induced by Leishmania major in mice requires a functional Fas (APO-1, CD95) pathway of cytotoxicity. Eur. J. Immunol. 28, 237-245.

Cornberg, M., Clute, S.C., Watkin, L.B., Saccoccio, F.M., Kim, S., Naumov, Y.N., Brehm, M.A., Aslan, N., Welsh, R.M., and Selin, L.K. (2010). CD8 T Cell Cross-Reactivity Networks Mediate Heterologous Immunity in Human EBV and Murine Vaccinia Virus Infections. J. Immunol. 184, 2825-2838.

Cosman, D., Mullberg, J., Sutherland, C., Chin, W., Armitage, R., Fanslow, W., Kubin, M., and Chalupny, N. (2001). ULBPs, novel MHC class I-related molecules bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14, 123-133.

Couppie, P., Clyti, E., Sobesky, M., Bissuel, F., Del Giudice, P., Sainte-Marie, D., Dedet, J., Carme, B., and Pradinaud, R. (2004). Comparative study of cutaneous leishmaniasis in human immunodeficiency virus (HIV)-infected patients and non-HIV-infected patients in French Guiana. Br. J. Dermatol. 151, 1165-1171.

Da-Cruz, A.M., Oliveira-Neto, M.P., Bertho, A.L., Mendes-Aguiar, C.O., and Coutinho, S.G. (2010). T Cells Specific to Leishmania and Other Nonrelated Microbial Antigens Can Migrate to Human Leishmaniasis Skin Lesions. J. Invest. Dermatol. 130, 1329-1336.

Da-Cruz, A., Conceicaosilva, F., Bertho, A., and Coutinho, S. (1994). Leishmania-Reactive CD4+ and CD8+ T-Cells Associated with Cure of Human Cutaneous Leishmaniasis. Infect. Immun. 62, 2614-2618.

Dantas, M.L., de Oliveira, J.C., Carvalho, L., Passos, S.T., Queiroz, A., Machado, P., Carvalho, E., and Arruda, S. (2013). CD8+T cells in situ in different clinical forms of human cutaneous leishmaniasis. Rev. Soc. Bras. Med. Trop. 46, 728-734.

Diefenbach, A., Jamieson, A., Liu, S., Shastri, N., and Raulet, D. (2000). Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat. Immunol. 1, 119-126.

Diefenbach, A., Tomasello, E., Lucas, M., Jamieson, A., Hsia, J., Vivier, E., and Raulet, D. (2002). Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. Nat. Immunol. 3, 1142-1149.

DiSpirito, J.R., and Shen, H. (2010). Quick to remember, slow to forget: rapid recall responses of memory CD8(+) T cells. Cell Res. 20, 13-23.

Dudda, J.C., Perdue, N., Bachtanian, E., and Campbell, D.J. (2008). Foxp3(+) regulatory T cells maintain immune homeostasis in the skin. J. Exp. Med. 205, 1559-1565.

Effros, R.B., Doherty, P.C., Gerhard, W., and Bennink, J. (1977). Generation of both Cross-Reactive and Virus-Specific T-Cell Populations After Immunization with Serologically Distinct Influenza-a Viruses. J. Exp. Med. 145, 557-568.

Ehl, S., Hombach, J., Aichele, P., Hengartner, H., and Zinkernagel, R. (1997). Bystander activation of cytotoxic T cells: Studies on the mechanism and evaluation of in vivo significance in a transgenic mouse model. J. Exp. Med. 185, 1241-1251.

Ehrlich, L.I.R., Ogasawara, K., Hamerman, J.A., Takaki, R., Zingoni, A., Allison, J.P., and Lanier, L.L. (2005). Engagement of NKG2D by Cognate Ligand or Antibody Alone Is Insufficient to Mediate Costimulation of Human and Mouse CD8+ T Cells. J. Immunol. 174, 1922-1931.

Eisert, V., Munster, U., Simon, M.M., and Moll, H. (2002). The course of Leishmania major infection in mice lacking granzyme-mediated mechanisms. Immunobiology 205, 314-320.

Ely, K.H., Cauley, L.S., Roberts, A.D., Brennan, J.W., Cookenham, T., and Woodland, D.L. (2003). Nonspecific Recruitment of Memory CD8+ T Cells to the Lung Airways During Respiratory Virus Infections. J. Immunol. 170, 1423-1429.

Farah, F.S., and Malak, J.A. (1971). Cutaneous Leishmaniasis. Arch. Dermatol. 103, 467-&.

Faria, D.R., Souza, P.E.A., Duraes, F.V., Carvalho, E.M., Gollob, K.J., Machado, P.R., and Dutra, W.O. (2009). Recruitment of CD8(+) T cells expressing granzyme A is associated with lesion progression in human cutaneous leishmaniasis. Parasite Immunol. 31, 432-439.

Foulds, K.E., and Shen, H. (2006). Clonal Competition Inhibits the Proliferation and Differentiation of Adoptively Transferred TCR Transgenic CD4 T Cells in Response to Infection. J. Immunol. 176, 3037-3043.

Freeman, B.E., Hammarlund, E., Raué, H., and Slifka, M.K. (2012). Regulation of innate CD8+ T-cell activation mediated by cytokines. Proc. Natl. Acad. Sci. 109, 9971-9976.

Gebhardt, T., Wakim, L.M., Eidsmo, L., Reading, P.C., Heath, W.R., and Carbone, F.R. (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat. Immunol. 10, 524-530.

Gebhardt, T., Whitney, P.G., Zaid, A., Mackay, L.K., Brooks, A.G., Heath, W.R., Carbone, F.R., and Mueller, S.N. (2011). Different patterns of peripheral migration by memory CD4(+) and CD8(+) T cells. Nature 477, 216-219.

Girardi, M., Oppenheim, D., Steele, C., Lewis, J., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R., and Hayday, A. (2001). Regulation of cutaneous malignancy by gamma delta T cells. Science 294, 605-609.

Gonzalez-Lombana, C., Gimblet, C., Bacellar, O., Oliveira, W.W., Passos, S., Carvalho, L.P., Goldschmidt, M., Carvalho, E.M., and Scott, P. (2013). IL-17 Mediates Immunopathology in the Absence of IL-10 Following *Leishmania major* Infection. PLoS Pathog. 9, 1-13.

Gray, J.M., and Cohn, D.L. (2013). Tuberculosis and HIV Coinfection Preface. Semin. Respir. Crit. Care Med. 34, 32-43.

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., and Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc. Natl. Acad. Sci. U.S.A. 93, 12445-12450.

Groh, V., Wu, J., Yee, C., and Spies, T. (2002). Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature 419, 734-738.

Gudjonsson, J.E., Johnston, A., Sigmundsdottir, H., and Valdimarsson, H. (2004). Immunopathogenic mechanisms in psoriasis. Clinical & Experimental Immunology 135, 1-8.

Guerra, N., Tan, Y.X., Joncker, N.T., Choy, A., Gallardo, F., Xiong, N., Knoblaugh, S., Cado, D., Greenberg, N.R., and Raulet, D.H. (2008). NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 28, 571-580.

Haanen, J.B.A.G., Wolkers, M.C., Kruisbeek, A.M., and Schumacher, T.N.M. (1999). Selective Expansion of Cross-Reactive Cd8+ Memory T Cells by Viral Variants. J. Exp. Med. 190, 1319-1328.

Hansen, C.H.F., Holm, T.L., Krych, F, Andresen, L., Nielsen, D.S., Rune, I., Hansen, A.K., and Skov, S. (2013). Gut microbiota regulates NKG2D ligand expression on intestinal epithelial cells. Eur. J. Immunol. 43, 447-457.

Harrington, L.E., Janowski, K.M., Oliver, J.R., Zajac, A.J., and Weaver, C.T. (2008). Memory CD4 T cells emerge from effector T-cell progenitors. Nature 452, 356-U8.

Harris, J.E., Harris, T.H., Weninger, W., Wherry, E.J., Hunter, C.A., and Turka, L.A. (2012). A Mouse Model of Vitiligo with Focused Epidermal Depigmentation Requires IFN-gamma for Autoreactive CD8(+) T-Cell Accumulation in the Skin. J. Invest. Dermatol. 132, 1869-1876.

Heinzel, F.P., Sadick, M.D., Holaday, B.J., Coffman, R.L., and Locksley, R.M. (1989). Reciprocal Expression of Interferon-Gamma Or Interleukin-4 during the Resolution Or Progression of Murine Leishmaniasis - Evidence for Expansion of Distinct Helper T-Cell Subsets. J. Exp. Med. 169, 59-72.

Heinzel, F.P., Schoenhaut, D.S., Rerko, R.M., Rosser, L.E., and Gately, M.K. (1993). Recombinant Interleukin-12 Cures Mice Infected with *Leishmania major*. J. Exp. Med. 177, 1505-1509.

Hermans, I.F., Silk, J.D., Yang, J., Palmowski, M.J., Gileadi, U., McCarthy, C., Salio, M., Ronchese, F., and Cerundolo, V. (2004). The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. J. Immunol. Methods 285, 25-40.

Hijnen, D., Knol, E.F., Gent, Y.Y., Giovannone, B., Beijn, S.J.P., Kupper, T.S., Bruijnzeel-Koomen, C.A.F.M., and Clark, R.A. (2013). CD8(+) T Cells in the Lesional Skin of Atopic Dermatitis and Psoriasis Patients Are an Important Source of IFN-gamma, IL-13, IL-17, and IL-22. J. Invest. Dermatol. 133, 973-979.

Hill, J.O., Awwad, M., and North, R.J. (1989). Elimination of CD4+ Suppressor T-Cells from Susceptible Balb/c Mice Releases CD8+ Lymphocyte-T to Mediate Protective Immunity Against Leishmania. J. Exp. Med. 169, 1819-1827.

Hogan, L.H., Co, D.O., Karman, J., Heninger, E., Suresh, M., and Sandor, M. (2007). Virally Activated CD8 T Cells Home to Mycobacterium bovis BCG-Induced Granulomas but Enhance Antimycobacterial Protection Only in Immunodeficient Mice. Infection and Immunity 75, 1154-1166.

Huber, M., Timms, E., Mak, T., Rollinghoff, M., and Lohoff, M. (1998). Effective and long-lasting immunity against the parasite Leishmania major in CD8-deficient mice. Infect. Immun. 66, 3968-3970.

Iser, D.M., and Sasadeusz, J.J. (2008). Current treatment of HIV/hepatitis B virus coinfection. J. Gastroenterol. Hepatol. 23, 699-706.

Jamieson, A.M., Diefenbach, A., McMahon, C.W., Xiong, N., Carlyle, J.R., and Raulet, D.H. (2002). The Role of the NKG2D Immunoreceptor in Immune Cell Activation and Natural Killing. Immunity 17, 19-29.

Jamieson, A.M., Pasman, L., Yu, S., Gamradt, P., Homer, R.J., Decker, T., and Medzhitov, R. (2013). Role of Tissue Protection in Lethal Respiratory Viral-Bacterial Coinfection. Science 340, 1230-1234.

Jones, W.T., Menna, J.H., and Wennerstrom, D.E. (1983). Lethal Synergism Induced in Mice by Influenza Type-a Virus and Type-Ia Group-B Streptococci. Infect. Immun. 41, 618-623.

Kaech, S.M., Hemby, S., Kersh, E., and Ahmed, R. (2002). Molecular and Functional Profiling of Memory CD8 T Cell Differentiation. Cell 111, 837-851.

Kamath, A., Sheasby, C., and Tough, D. (2005). Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. J. Immunol. 174, 767-776.

Kambayashi, T., Assarsson, E., Lukacher, A.E., Ljunggren, H., and Jensen, P.E. (2003). Memory CD8+ T Cells Provide an Early Source of IFN-γ. J. Immunol. 170, 2399-2408.

Karimi, M., Cao, T., Baker, J., Verneris, M., Soares, L., and Negrin, R. (2005). Silencing human NKG2D, DAP10, and DAP12 reduces cytotoxicity of activated CD8(+) T cells and NK cells. J. Immunol. 175, 7819-7828.

Kaye, P., and Scott, P. (2011). Leishmaniasis: complexity at the host-pathogen interface. Nat. Rev. Micro. 9, 604-615.

Kedzierski, L. (2010). Leishmaniasis Vaccine: Where are We Today? J. Glob. Inf. Dis. 2, 177-185.

Kellina, O.I. (1973). Differences in the sensitivity of inbred mice of different lines to *Leishmania tropica major*. Med. Parazitol. 42, 279-285.

Kersh, E.N., Kaech, S.M., Onami, T.M., Moran, M., Wherry, E.J., Miceli, M.C., and Ahmed, R. (2003). TCR signal transduction in antigen-specific memory CD8 T cells. J. Immunol. 170, 5455-5463.

Kim, S., Brehm, M., Welsh, R., and Selin, L. (2002). Dynamics of memory T cell proliferation under conditions of heterologous immunity and bystander stimulation. J. Immunol. 169, 90-98.

Kim, S., Cornberg, M., Wang, X.Z., Chen, H.D., Selin, L.K., and Welsh, R.M. (2005). Private specificities of CD8 T cell responses control patterns of heterologous immunity. J. Exp. Med. 201, 523-533.

Kimblin, N., Peters, N., Debrabant, A., Secundino, N., Egen, J., Lawyer, P., Fay, M.P., Kamhawi, S., and Sacks, D. (2008). Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. Proc. Natl. Acad. Sci. U.S.A. 105, 10125-10130.

Kobayashi, Y. (2008). The role of chemokines in neutrophil biology. Frontiers in Bioscience-Landmark 13, 2400-2407.

Kohlmeier, J.E., Cookenham, T., Roberts, A.D., Miller, S.C., and Woodland, D.L. (2010). Type I Interferons Regulate Cytolytic Activity of Memory CD8+ T Cells in the Lung Airways during Respiratory Virus Challenge. Immunity 33, 96-105.

Kohlmeier, J.E., Miller, S.C., and Woodland, D.L. (2007). Cutting Edge: Antigen Is Not Required for the Activation and Maintenance of Virus-Specific Memory CD8+ T Cells in the Lung Airways. J. Immunol. 178, 4721-4725.

Kolbaum, J., Ritter, U., Zimara, N., Brewig, N., Eschbach, M., and Breloer, M. (2011). Efficient control of Leishmania and Strongyloides despite partial suppression of nematode-induced Th2 response in co-infected mice. Parasite Immunol. 33, 226-235.

La Flamme, A., Scott, P., and Pearce, E. (2002). Schistosomiasis delays lesion resolution during Leishmania major infection by impairing parasite killing by macrophages. Parasite Immunol. 24, 339-345.

Lambotin, M., Barth, H., Moog, C., Habersetzer, F., Baumert, T.F., Stoll-Keller, F., and Fafi-Kremer, S. (2012). Challenges for HCV vaccine development in HIV-HCV coinfection. Expert Rev. Vaccines 11, 791-804.

Lang, P.A., Lang, K.S., Xu, H.C., Grusdat, M., Parish, I.A., Recher, M., Elford, A.R., Dhanji, S., Shaabani, N., Tran, C.W.*, et al.* (2012). Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. Proc. Natl. Acad. Sci. U.S.A. 109, 1210-1215.

Laskay, T., van Zandbergen, G., and Solbach, W. (2003). Neutrophil granulocytes - Trojan horses for *Leishmania major* and other intracellular microbes. Trends Microbiol. 11, 210-214.

Lertmemongkolchai, G., Cai, G.F., Hunter, C.A., and Bancroft, G.J. (2001). Bystander activation of CD8(+) T cells contributes to the rapid production of IFN-gamma in response to bacterial pathogens. J. Immunol. 166, 1097-1105.

Lessa, M.M., Lessa, H.A., Castro, T.W.N., Oliveira, A., Scherifer, A., Machado, P., and Carvalho, E.M. (2007). Mucosal leishmaniasis: epidemiological and clinical aspects. Braz. J. Otorhinolaryngol 73, 843-847.

Liu, K., Catalfamo, M., Li, Y., Henkart, P., and Weng, N. (2002). IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8(+) memory T cells. Proc. Natl. Acad. Sci. U.S.A. 99, 6192-6197.

Liu, L., Fuhlbrigge, R.C., Karibian, K., Tian, T., and Kupper, T.S. (2006). Dynamic programing of CD8(+) T cell trafficking after live viral immunization. Immunity 25, 511-520.

Locksley, R.M., Heinzel, F.P., Fankhauser, J.E., Nelson, C.S., and Sadick, M.D. (1988). Cutaneous Host Defense in Leishmaniasis - Interaction of Isolated Dermal Macrophages and Epidermal Langerhans Cells with the Insect-Stage Promastigote. Infect. Immun. 56, 336-342.

Mackay, L.K., Rahimpour, A., Ma, J.Z., Collins, N., Stock, A.T., Hafon, M., Vega-Ramos, J., Lauzurica, P., Mueller, S.N., Stefanovic, T.*, et al.* (2013). The developmental pathway for CD103(+)CD8(+) tissue-resident memory T cells of skin. Nat. Immunol. 14, 1294-+.

Mackay, L.K., Stock, A.T., Ma, J.Z., Jones, C.M., Kent, S.J., Mueller, S.N., Heath, W.R., Carbone, F.R., and Gebhardt, T. (2012). Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. Proc. Natl. Acad. Sci. U.S.A. 109, 7037-7042.

Malarkannan, S., Shih, P., Eden, P., Horng, T., Zuberi, A., Christianson, G., Roopenian, D., and Shastri, N. (1998). The molecular and functional characterization of a dominant minor H antigen, H60. J. Immunol. 161, 3501-3509.

Markiewicz, M.A., Carayannopoulos, L.N., Naidenko, O.V., Matsui, K., Burack, W.R., Wise, E.L., Fremont, D.H., Allen, P.M., Yokoyama, W.M., Colonna, M., and Shaw, A.S. (2005). Costimulation through NKG2D Enhances Murine CD8+ CTL Function: Similarities and Differences between NKG2D and CD28 Costimulation. J. Immunol. 175, 2825-2833.

Markiewicz, M., Wise, E., Buchwald, Z., Pinto, A., Zafirova, B., Polic, B., and Shaw, A. (2012). RAE1ε Ligand Expressed on Pancreatic Islets Recruits NKG2D Receptor-Expressing Cytotoxic T Cells Independent of T Cell Receptor Recognition. Immunity 36, 132-141.

Maroli, M., Feliciangeli, M.D., Bichaud, L., Charrel, R.N., and Gradoni, L. (2013). Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Med. Vet. Entomol. 27, 123-147.

Marshall, H.D., Prince, A.L., Berg, L.J., and Welsh, R.M. (2010). IFN-alpha beta and Self-MHC Divert CD8 T Cells into a Distinct Differentiation Pathway Characterized by Rapid Acquisition of Effector Functions. J. Immunol. 185, 1419-1428.

Marshall, H.D., Urban, S.L., and Welsh, R.M. (2011). Virus-Induced Transient Immune Suppression and the Inhibition of T Cell Proliferation by Type I Interferon. J. Virol. 85, 5929-5939.

Masopust, D., Vezys, V., Usherwood, E.J., Cauley, L.S., Olson, S., Marzo, A.L., Ward, R.L., Woodland, D.L., and Lefrancois, L. (2004). Activated primary and memory CD8 T cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. J. Immunol. 172, 4875-4882.

Masopust, D., Choo, D., Vezys, V., Wherry, E.J., Duraiswamy, J., Akondy, R., Wang, J., Casey, K.A., Barber, D.L., Kawamura, K.S.*, et al.* (2010). Dynamic T cell migration program provides resident memory within intestinal epithelium. J. Exp. Med. 207, 553-564.

Masopust, D., Murali-Krishna, K., and Ahmed, R. (2007). Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: It is even bigger than we thought. J. Virol. 81, 2002-2011.

Mathurin, K.S., Martens, G.W., Kornfeld, H., and Welsh, R.M. (2009). CD4 T-Cell-Mediated Heterologous Immunity between Mycobacteria and Poxviruses. J. Virol. 83, 3528-3539.

Mattner, F., Magram, J., Ferrante, J., Launois, P., Padova, K.D., Behin, R., Gately, M.K., Louis, J.A., and Alber, G. (1996). Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. Eur. J. Immunol. 26, 1553-1559.

Meresse, B., Chen, Z., Ciszewski, C., Tretiakova, M., Bhagat, G., Krausz, T.N., Raulet, D.H., Lanier, L.L., Groh, V., Spies, T.*, et al.* (2004). Coordinated Induction by IL15 of a

TCR-Independent NKG2D Signaling Pathway Converts CTL into Lymphokine-Activated Killer Cells in Celiac Disease. Immunity 21, 357-366.

Meresse, S., Steele-Mortimer, O., Moreno, E., Desjardins, M., Finlay, B., and Gorvel, J. (1999). Controlling the maturation of pathogen-containing vacuoles: a matter of life and death. Nat. Cell Biol. 1, E183-E188.

Metzger, D.W., and Sun, K. (2013). Immune Dysfunction and Bacterial Coinfections following Influenza. J. Immunol.191, 2047-2052.

Mims, C., and Wainwrig, S. (1968). Immunodepressive Action of Lymphocytic Choriomeningitis Virus in Mice. J. Immunol. 101, 717-&.

Mongkolsapaya, J., Dejnirattisai, W., Xu, X., Vasanawathana, S., Tangthawornchaikul, N., Chairunsri, A., Sawasdivorn, S., Duangchinda, T., Dong, T., Rowland-Jones, S.*, et al.* (2003). Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat. Med. 9, 921-927.

Moran, A.E., Holzapfel, K.L., Xing, Y., Cunningham, N.R., Maltzman, J.S., Punt, J., and Hogquist, K.A. (2011). T cell receptor signal strength in T-reg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. J. Exp. Med. 208, 1279-1289.

Morens, D.M., Taubenberger, J.K., and Fauci, A.S. (2008). Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: Implications for pandemic influenza preparedness. J. Infect. Dis. 198, 962-970.

Mosmann, T., Cherwinski, H., Bond, M., Giedlin, M., and Coffman, R. (1986). 2 Types of Murine Helper T-Cell Clone: Definition According to Profiles of Lymphokine Activities and Secreted Proteins. J. Immunol. 136, 2348-2357.

Muller, I., Kropf, P., Etges, R.J., and Louis, J.A. (1993). Gamma interferon response in secondary *Leishmania major* infection: role of CD8+ T cells. Infect. Immun. 61, 3730-3738.

Muller, I., Pedrazzini, T., Kropf, P., Louis, J., and Milon, G. (1991). Establishment of Resistance to *Leishmania major* Infection in Susceptible Balb/c Mice Requires Parasite-Specific CD8+ T-Cells. Int. Immunol. 3, 587-597.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J.D., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting Antigen-Specific CD8 T Cells: A Reevaluation of Bystander Activation during Viral Infection. Immunity 8, 177-187.

Murray, H.W., Berman, J.D., Davies, C.R., and Saravia, N.G. (2005). Advances in leishmaniasis. Lancet 366, 1561-1577.

Nahill, S.R., and Welsh, R.M. (1993). High frequency of cross-reactive cytotoxic T lymphocytes elicited during the virus-induced polyclonal cytotoxic T lymphocyte response. The Journal of Experimental Medicine 177, 317-327.

Naik, S., Bouladoux, N., Wilhelm, C., Molloy, M.J., Salcedo, R., Kastenmuller, W., Deming, C., Quinones, M., Koo, L., Conlan, S.*, et al.* (2012). Compartmentalized Control of Skin Immunity by Resident Commensals. Science 337, 1115-1119.

Narni-Mancinelli, E., Campisi, L., Bassand, D., Cazareth, J., Gounon, P., Glaichenhaus, N., and Lauvau, G. (2007). Memory CD8(+) T cells mediate antibacterial immunity via CCL3 activation of TNF/ROI+ phagocytes. J. Exp. Med. 204, 2075-2087.

Nasseri, M., and Modabber, F.Z. (1979). Generalized Infection and Lack of Delayed-Hypersensitivity in Balb-C Mice Infected with *Leishmania-Tropica Major.* Infect. Immun. 26, 611-614.

Newlove, T., Guimaraes, L.H., Morgan, D.J., Alcantara, L., Glesby, M.J., Carvalho, E.M., and Machado, P.R. (2011). Antihelminthic Therapy and Antimony in Cutaneous Leishmaniasis: A Randomized, Double-Blind, Placebo-Controlled Trial in Patients Co-Infected with Helminths and *Leishmania braziliensis*. Am. J. Trop. Med. Hyg. 84, 551-555.

Ng, L.G., Hsu, A., Mandell, M.A., Roediger, B., Hoeller, C., Mrass, P., Iparraguirre, A., Cavanagh, L.L., Triccas, J.A., Beverley, S.M., Scott, P., and Weninger, W. (2008). Migratory Dermal Dendritic Cells Act as Rapid Sensors of Protozoan Parasites. Plos Pathogens 4, e1000222.

Nice, T.J., Coscoy, L., and Raulet, D.H. (2009). Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. J. Exp. Med. 206, 287-298.

Nie, S., Lin, S., Kim, S., Welsh, R.M., and Selin, L.K. (2010). Pathological Features of Heterologous Immunity Are Regulated by the Private Specificities of the Immune Repertoire. Am. J. Pathol. 176, 2107-2112.

Noazin, S., Modabber, F., Khamesipour, A., Smith, P.G., Moulton, L.H., Nasseri, K., Sharifi, I., Khalil, E.A.G., Bernal, I.D.V., Antunes, C.M.F., Kieny, M.P., and Tanner, M. (2008). First generation leishmaniasis vaccines: A review of field efficacy trials. Vaccine 26, 6759-6767.

Nomura, M., Takihara, Y., and Shimada, K. (1994). Isolation and Characterization of Retinoic Acid-Inducible Cdna Clones in F9 Cells - One of the Early Inducible Clones Encodes a Novel Protein Sharing several Highly Homologous Regions with a Drosophila-Polyhomeotic Protein. Differentiation 57, 39-50.

Novais, F.O., Carvalho, L.P., Graff, J.W., Beiting, D.P., Ruthel, G., Roos, D.S., Betts, M.R., Goldschmidt, M.H., Wilson, M.E., de Oliveira, C.I., and Scott, P. (2013). Cytotoxic T Cells Mediate Pathology and Metastasis in Cutaneous Leishmaniasis. PLoS Pathog. 9, e1003504.

Odumade, O.A., Knight, J.A., Schmeling, D.O., Masopust, D., Balfour, H.H., and Hogquist, K.A. (2012). Primary Epstein-Barr virus infection does not erode preexisting CD8+ T cell memory in humans. J. Exp. Med. 209, 471-478.

Ogasawara, K., Hamerman, J.A., Ehrlich, L.R., Bour-Jordan, H., Santamaria, P., Bluestone, J.A., and Lanier, L.L. (2004). NKG2D Blockade Prevents Autoimmune Diabetes in NOD Mice. Immunity 20, 757-767.

Okamoto, S., Kawabata, S., Nakagawa, I., Okuno, Y., Goto, T., Sano, K., and Hamada, S. (2003). Influenza A virus-infected hosts boost an invasive type of Streptococcus pyogenes infection in mice. J. Virol. 77, 4104-4112.

Okwor, I., and Uzonna, J.E. (2013). The immunology of Leishmania/HIV co-infection. Immunol. Res. 56, 163-171.

Oliveira, C.F., Manzoni-de-Almeida, D., Mello, P.S., Natale, C.C., Santiago, H.d.C., Miranda, L.d.S., Ferraz, F.O., dos Santos, L.M., Teixeira, M.M., Esteves Arantes, R.M., and Vieira, L.Q. (2012). Characterization of Chronic Cutaneous Lesions from TNF-Receptor-1-Deficient Mice Infected by *Leishmania major*. Clin. Dev. Immunol. 1-12.

Oliveira, F., Bafica, A., Rosato, A.B., Favali, C.B.F., Costa, J.M., Cafe, V., Barral-Netto, M., and Barral, A. (2011). Lesion Size Correlates with Leishmania Antigen-Stimulated TNF-Levels in Human Cutaneous Leishmaniasis. Am. J. Trop. Med. Hyg. 85, 70-73.

O'Neal, S.E., Guimaraes, L.H., Machado, P.R., Alcantara, L., Morgan, D.J., Passos, S., Glesby, M.J., and Carvalho, E.M. (2007). Influence of helminth infections on the clinical course of and immune response to Leishmania braziliensis cutaneous leishmaniasis. J. Infect. Dis. 195, 142-148.

Ortega, C., Fernandez-A, S., Carrillo, J.M., Romero, P., Molina, I.J., Moreno, J.C., and Santamaria, M. (2009). IL-17-producing CD8(+) T lymphocytes from psoriasis skin plaques are cytotoxic effector cells that secrete Th17-related cytokines. J. Leukoc. Biol. 86, 435-443.

Ostler, T., Pircher, H., and Ehl, S. (2003). "Bystander" recruitment of systemic memory T cells delays the immune response to respiratory virus infection. Eur. J. Immunol. 33, 1839-1848.

Overath, P., and Harbecke, D. (1993). Course of Leishmania Infection in Beta-2-Microglobulin-Deficient Mice. Immunol. Lett. 37, 13-17.

Paschen, A., Sucker, A., Hill, B., Moll, I., Zapatka, M., Nguyen, X.D., Sim, G.C., Gutmann, I., Hassel, J., Becker, J.C*., et al.* (2009). Differential Clinical Significance of Individual NKG2D Ligands in Melanoma: Soluble ULBP2 as an Indicator of Poor Prognosis Superior to S100B. Clinical Cancer Research 15, 5208-5215.

Pearson, R.D., and Sousa, A.d.Q. (1996). Clinical Spectrum of Leishmaniasis. Clin. Infect. Dis. 22, 1-11.

Pegram, H.J., Andrews, D.M., Smyth, M.J., Darcy, P.K., and Kershaw, M.H. (2011). Activating and inhibitory receptors of natural killer cells. Immunol. Cell Biol. 89, 216-224.

Peltola, V.T., and McCullers, J.A. (2004). Respiratory viruses predisposing to bacterial infections: role of neuraminidase. Pediatr. Infect. Dis. J. 23, S87-S96.

Penn, D.J., Damjanovich, K., and Potts, W.K. (2002). MHC heterozygosity confers a selective advantage against multiple-strain infections. Proc. Natl. Acad. Sci. U.S.A. 99, 11260-11264.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M.P., Germain, R.N., and Sacks, D. (2008). In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321, 970-974.

Pircher, H., Burki, K., Lang, R., Hengartner, H., and Zinkernagel, R.M. (1989). Tolerance Induction in Double Specific T-Cell Receptor Transgenic Mice Varies with Antigen. Nature 342, 559-561.

Polley, R., Sanos, S.L., Prickett, S., Haque, A., and Kaye, P.M. (2005). Chronic *Leishmania donovani* Infection Promotes Bystander CD8+ Cell Expansion and Heterologous Immunity. Infect. Immun. 73, 7996-8001.

Prickett, S.P., Gray, P.M., Colpitts, S.L., Scott, P., Kaye, P.M., and Smith, D.F. (2006). In vivo recognition of ovalbumin expressed by transgenic Leishmania is determined by its subcellular localization. J. Immunol. 176, 4826-4833.

Raué, H., Beadling, C., Haun, J., and Slifka, M. (2013). Cytokine-Mediated Programmed Proliferation of Virus-Specific CD8+ Memory T Cells. Immunity 38, 131-139.

Raué, H., Brien, J.D., Hammarlund, E., and Slifka, M.K. (2004). Activation of Virus-Specific CD8+ T Cells by Lipopolysaccharide-Induced IL-12 and IL-18. J. Immunol. 173, 6873-6881.

Raulet, D. (2003). Roles of the NKG2D immunoreceptor and its ligands. Nat. Rev. Immunol. 3, 781-790.

Redford, P.S., Mayer-Barber, K.D., McNab, F.W., Stavropoulos, E., Wack, A., Sher, A., and O'Garra, A. (2014). Influenza A Virus Impairs Control of Mycobacterium tuberculosis Coinfection Through a Type I Interferon Receptor-Dependent Pathway. J. Infect. Dis. 209, 270-274.

Reiser, J., Darnault, C., Gregoire, C., Mosser, T., Mazza, G., Kearney, A., van der Merwe, P., Fontecilla-Camps, J., Housset, D., and Malissen, B. (2003). CDR3 loop flexibility contributes to the degeneracy of TCR recognition. Nat. Immunol. 4, 241-247.

Res, P.C.M., Piskin, G., de Boer, O.J., van der Loos, C.M., Teeling, P., Bos, J.D., and Teunissen, M.B.M. (2010). Overrepresentation of IL-17A and IL-22 Producing CD8 T Cells in Lesional Skin Suggests Their Involvement in the Pathogenesis of Psoriasis. Plos One 5, 1-11.

Rohousova, I., Ozensoy, S., Ozbel, Y., and Volf, P. (2005). Detection of species-specific antibody response of humans and mice bitten by sand flies. Parasitology 130, 493-499.

Rohousova, I., and Volf, P. (2006). Sand fly saliva: effects on host immune response and Leishmania transmission. Folia Parasitol. 53, 161-171.

Romero, G., Guerra, M., Paes, M., and Macedo, V. (2001). Comparison of cutaneous leishmaniasis due to *Leishmania (Viannia) braziliensis* and *L. (V.) Guyanensis* in Brazil: Therapeutic response to meglumine antimoniate. Am. J. Trop. Med. Hyg. 65, 456-465.

Rosen, D., Araki, M., Hamerman, J., Chen, T., Yamamura, T., and Lanier, L. (2004). A structural basis for the association of DAP12 with mouse, but not human, NKG2D. J. Immunol. 173, 2470-2478.

Russell, D., Xu, S., and Chakraborty, P. (1992). Intracellular Trafficking and the Parasitophorous Vacuole of *Leishmania-Mexicana*-Infected Macrophages. J. Cell. Sci. 103, 1193-1210.

Sacks, D., and Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to Leishmania major in mice. Nat. Rev. Immunol. 2, 845-858.

Santiago, H.C., Oliveira, M.A.P., Bambirra, E.A., Faria, A.M.C., Afonso, L.C.C., Vieira, L.Q., and Gazzinelli, R.T. (1999). Coinfection with Toxoplasma gondii inhibits antigen-specific Th2 immune responses, tissue inflammation, and parasitism in BALB/c mice infected with Leishmania major. Infect. Immun. 67, 4939-4944.

Santos, C.d.S., Boaventura, V., Cardoso, C.R., Tavares, N., Lordelo, M.J., Noronha, A., Costa, J., Borges, V.M., de Oliveira, C.I., Van Weyenbergh, J.*, et al.* (2013). CD8+Granzyme B+ mediated tissue injury versus CD4+IFNg+ mediated parasite killing in human cutaneous leishmaniasis. J. Invest. Dermatol. 133, 1533-1540.

Saron, M.F., Shidani, B., Nahori, M.A., Guillon, J.C., and Truffabachi, P. (1990). Lymphocytic Choriomeningitis Virus-Induced Immunodepression - Inherent Defect of Lymphocyte-B and Lymphocyte-T. J. Virol. 64, 4076-4083.

Schenkel, J.M., Fraser, K.A., Vezys, V., and Masopust, D. (2013). Sensing and alarm function of resident memory CD8+ T cells. Nat. Immunol. 14, 509-513.

Sckisel, G.D., Tietze, J.K., Zamora, A.E., Hsiao, H., Priest, S.O., Wilkins, D.E.C., Lanier, L.L., Blazar, B.R., Baumgarth, N., and Murphy, W.J. (2014). Influenza infection results in local expansion of memory CD8+T cells with antigen non-specific phenotype and function. Clinical & Experimental Immunology 175, 79-91.

Scott, P. (1991). IFN-Gamma Modulates the Early Development of Th1 and Th2 Responses in a Murine Model of Cutaneous Leishmaniasis. J. Immunol. 147, 3149-3155.

Scott, P., Natovitz, P., Coffman, R.L., Pearce, E., and Sher, A. (1988). Immunoregulation of Cutaneous Leishmaniasis - T-Cell Lines that Transfer Protective Immunity Or Exacerbation Belong to Different T-Helper Subsets and Respond to Distinct Parasite Antigens. J. Exp. Med. 168, 1675-1684.

Scott, P., Artis, D., Uzonna, J., and Zaph, C. (2004). The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development. Immunol. Rev. 201, 318-338.

Segel, G.B., Halterman, M.W., and Lichtman, M.A. (2011). The paradox of the neutrophil's role in tissue injury. Journal of Leukocyte Biology 89, 359-372.

Selin, L.K., Brehm, M.A., Naumov, Y.N., Cornberg, M., Kim, S., Clute, S.C., and Welsh, R.M. (2006). Memory of mice and men: CD8+ T-cell cross-reactivity and heterologous immunity. Immunol. Rev. 211, 164-181.

Selin, L.K., Varga, S.M., Wong, I.C., and Welsh, R.M. (1998). Protective Heterologous Antiviral Immunity and Enhanced Immunopathogenesis Mediated by Memory T Cell Populations. J. Exp. Med. 188, 1705-1715.

Sharma, S., and Thomas, P.G. (2014). The two faces of heterologous immunity: protection or immunopathology. Journal of Leukocyte Biology 95, 405-416.

Sheil, J.M., Bevan, M.J., and Lefrancois, L. (1987). Characterization of dual-reactive H-2Kb-restricted anti-vesicular stomatitus virus and alloreactive cytotoxic T cells. J. Immunol. 138, 3654-3660.

Singh, B., and Sundar, S. (2012). Leishmaniasis: Vaccine candidates and perspectives. Vaccine 30, 3834-3842.

Smith, L.E., Rodrigues, M., and Russell, D.G. (1991). The interaction between CD8+ cytotoxic T cells and Leishmania-infected macrophages. J. Exp. Med. 174, 499-505.

Soudja, S., Ruiz, A., Marie, J., and Lauvau, G. (2012). Inflammatory Monocytes Activate Memory CD8+ T and Innate NK Lymphocytes Independent of Cognate Antigen during Microbial Pathogen Invasion. Immunity 37, 549-562.

Späth, G.F., and Beverley, S.M. (2001). A Lipophosphoglycan-Independent Method for Isolation of Infective Leishmania Metacyclic Promastigotes by Density Gradient Centrifugation. Exp. Parasitol. 99, 97-103.

Starr, T., Jameson, S., and Hogquist, K. (2003). Positive and negative selection of T cells. Annu. Rev. Immunol. 21, 139-176.

Stelekati, E., and Wherry, E. . (2012). Chronic Bystander Infections and Immunity to Unrelated Antigens. Cell Host Microbe 12, 458-469.

Stevceva, L., Alvarez, X., Lackner, A.A., Tryniszewska, E., Kelsall, B., Nacsa, J., Tartaglia, J., Strober, W., and Franchini, G. (2002). Both Mucosal and Systemic Routes of Immunization with the Live, Attenuated NYVAC/Simian Immunodeficiency Virus SIVgpe Recombinant Vaccine Result in Gag-Specific CD8+ T-Cell Responses in Mucosal Tissues of Macaques. J. Virol. 76, 11659-11676.

Stonier, S.W., and Schluns, K.S. (2010). Trans-presentation: A novel mechanism regulating IL-15 delivery and responses. Immunol. Lett. 127, 85-92.

Swihart, K., Fruth, U., Messmer, N., Hug, K., Behin, R., Huang, S., Delgiudice, G., Aguet, M., and Louis, J.A. (1995). Mice from a Genetically Resistant Background Lacking the Interferon-Gamma Receptor are Susceptible to Infection with Leishmania-Major but Mount a Polarized T-Helper Cell 1-Type CD4(+) T-Cell Response. J. Exp. Med. 181, 961-971.

Sypek, J.P., Chung, C.L., Mayor, S.E.H., Subramanyam, J.M., Goldman, S.J., Sieburth, D.S., Wolf, S.F., and Schaub, R.G. (1993). Resolution of Cutaneous Leishmaniasis - Interleukin-12 Initiates a Protective T-Helper Type-1 Immune-Response. J. Exp. Med. 177, 1797-1802.

Tabbara, K.S., Al-Azmi, A.S., and Al Salem, J.A. (2004). Coinfection with Listeria monocytogenes potentiates the response of BALB/c mice against *Leishmania major*. J. Egypt. Soc. Parasitol. 34, 349-66.

Takada, A., Yoshida, S., Kajikawa, M., Miyatake, Y., Tomaru, U., Sakai, M., Chiba, H., Maenaka, K., Kohda, D., Fugo, K., and Kasahara, M. (2008). Two Novel NKG2D Ligands of the Mouse H60 Family with Differential Expression Patterns and Binding Affinities to NKG2D. J. Immunol. 180, 1678-1685.

Tietze, J.K., Wilkins, D.E.C., Sckisel, G.D., Bouchlaka, M.N., Alderson, K.L., Weiss, J.M., Ames, E., Bruhn, K.W., Craft, N., Wiltrout, R.H.*, et al.* (2012). Delineation of antigen-specific and antigen-nonspecific CD8+ memory T-cell responses after cytokine-based cancer immunotherapy. Blood 119, 3073-3083.

Titus, R., Milon, G., Marchal, G., Vassalli, P., Cerottini, J., and Louis, J. (1987). Involvement of Specific Lyt-2+ T-Cells in the Immunological Control of Experimentally Induced Murine Cutaneous Leishmaniasis. Eur. J. Immunol. 17, 1429-1433.

Tough, D.F., Borrow, P., and Sprent, J. (1996). Induction of Bystander T Cell Proliferation by Viruses and Type I Interferon in Vivo. Science 272, 1947-1950.

Tough, D., and Sprent, J. (1998). Anti-viral immunity: Spotting virus-specific T cells. Curr. Biol. 8, R498-R501.

Urbani, S., Amadei, B., Fisicaro, P., Pilli, M., Missale, G., Bertoletti, A., and Ferrari, C. (2005). Heterologous T cell immunity in severe hepatitis C virus infection. J. Exp. Med. 201, 675-680.

Uzonna, J.E., Joyce, K.L., and Scott, P. (2004). Low Dose Leishmania major Promotes a Transient T Helper Cell Type 2 Response That Is Down-regulated by Interferon γ–producing CD8+ T Cells. J. Exp. Med. 199, 1559-1566.

Varadarajan, N., Julg, B., Yamanaka, Y.J., Chen, H., Ogunniyi, A.O., McAndrew, E., Porter, L.C., Piechocka-Trocha, A., Hill, B.J., Douek, D.C.*, et al.* (2011). A high-throughput single-cell analysis of human CD8(+) T cell functions reveals discordance for cytokine secretion and cytolysis. J. Clin. Invest. 121, 4322-4331.

Verneris, M.R., Karami, M., Baker, J., Jayaswal, A., and Negrin, R.S. (2004). Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. Blood 103, 3065-3072.

Vezys, V., Yates, A., Casey, K.A., Lanier, G., Ahmed, R., Antia, R., and Masopust, D. (2009). Memory CD8 T-cell compartment grows in size with immunological experience. Nature 457, 196-199.

Wack, A., Openshaw, P., and O'Garra, A. (2011). Contribution of cytokines to pathology and protection in virus infection. Current Opinion in Virology 1, 184-195.

Wang, Z.E., Reiner, S.L., Hatam, F., Heinzel, F.P., Bouvier, J., Turck, C.W., and Locksley, R.M. (1993). Targeted Activation of CD8 Cells and Infection of Beta-2-Microglobulin-Deficient Mice Fail to Confirm a Primary Protective Role for CD8 Cells in Experimental Leishmaniasis. J. Immunol. 151, 2077-2086.

Wedemeyer, H., Mizukoshi, E., Davis, A., Bennink, J., and Rehermann, B. (2001). Cross-reactivity between hepatitis C virus and influenza A virus determinant-specific cytotoxic T cells. J. Virol. 75, 11392-11400.

Welsh, R.M., Selin, L.K., and Szomolanyi-Tsuda, E. (2004). Immunological memory to viral infections. Annu. Rev. Immunol. 22, 711-743.

Wherry, E.J., Teichgraber, V., Becker, T.C., Masopust, D., Kaech, S.M., Antia, R., von Andrian, U.H., and Ahmed, R. (2003). Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. 4, 225-234.

Wilson, E.B., Yamada, D.H., Elsaesser, H., Herskovitz, J., Deng, J., Cheng, G., Aronow, B.J., Karp, C.L., and Brooks, D.G. (2013). Blockade of Chronic Type I Interferon Signaling to Control Persistent LCMV Infection. Science 340, 202-207.

Wirth, T.C., Martin, M.D., Starbeck-Miller, G., Harty, J.T., and Badovinac, V.P. (2011). Secondary CD8+ T-cell responses are controlled by systemic inflammation. Eur. J. Immunol. 41, 1321-1333.

Wlodarczyk, M.F., Kraft, A.R., Chen, H.D., Kenney, L.L., and Selin, L.K. (2013). Anti-IFN-gamma and Peptide-Tolerization Therapies Inhibit Acute Lung Injury Induced by Cross-Reactive Influenza A-Specific Memory T Cells. Journal of Immunology 190, 2736-2746.

World Health Organization. (2014). Leishmaniasis. http://www.who.int/leishmaniasis/burden/en/

Yajima, T., Nishimura, H., Sad, S., Shen, H., Kuwano, H., and Yoshikai, Y. (2005). A Novel Role of IL-15 in Early Activation of Memory CD8+ CTL after Reinfection. J. Immunol. 174, 3590-3597.

Yoshida, S., Mohamed, R.H., Kajikawa, M., Koizumi, J., Tanaka, M., Fugo, K., Otsuka, N., Maenaka, K., Yagita, H., Chiba, H., and Kasahara, M. (2012). Involvement of an NKG2D Ligand H60c in Epidermal Dendritic T Cell-Mediated Wound Repair. J. Immunol. 188, 3972-3979.

Yoshimoto, T., Takeda, K., Tanaka, T., Ohkusu, K., Kashiwamura, S., Okamura, H., Akira, S., and Nakanishi, K. (1998). IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: Synergism with IL-18 for IFN-gamma production. J. Immunol. 161, 3400-3407.

Zafirova, B., Wensveen, F.M., Gulin, M., and Polic, B. (2011). Regulation of immune cell function and differentiation by the NKG2D receptor. Cell Mol. Life Sci. 68, 3519-3529.

Zhao, Z., Granucci, F., Yeh, L., Schaffer, P.A., and Cantor, H. (1998). Molecular Mimicry by Herpes Simplex Virus-Type 1: Autoimmune Disease After Viral Infection. Science 279, 1344-1347.

Zou, Z., Nomura, M., Takihara, Y., Yasunaga, T., and Shimada, K. (1996). Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: A novel cDNA family encodes cell surface proteins sharing partial homology with MHC class I molecules. J. Biochem. 119, 319-328.