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**HETEROLOGOUS IMMUNITY AND T CELL STABILITY DURING VIRAL
INFECTIONS**

A Dissertation Presented

By

JENNY WUN-YUE CHE

**Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of**

DOCTOR OF PHILOSOPHY

February 10, 2014

IMMUNOLOGY AND VIROLOGY PROGRAM

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By

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Abstract

The immune response to an infection is determined by a number of factors, which also affect the generation of memory T cells afterwards. The immune response can also affect the stability of the pre-existing memory populations. The memory developed after an infection can influence the response to subsequent infections with unrelated pathogens. This heterologous immunity may deviate the course of disease and alter the disease outcome. The generation and stability of memory CD8 T cells and the influence of the history of infections on subsequent heterologous infections are studied in this thesis using different viral infection sequences.

Previous studies using mice lacking individual immunoproteasome catalytic subunits showed only modest alterations in the CD8 T cell response to lymphocytic choriomeningitis virus (LCMV). In this study, I found that the CD8 T cell response to LCMV was severely impaired in mice lacking all three catalytic subunits of the immunoproteasome, altering the immunodominance hierarchy of the CD8 T cell response and CD8 T cell memory. Adoptive transfer experiments suggested that both inefficient antigen presentation and altered T cell repertoire contribute to the reduction of the CD8 T cell response in the immunoproteasome knockout mice.

Immune responses generated during infections can reduce pre-existing memory T cell populations. Memory CD8 T cells have been shown to be reduced by subsequent heterologous infections. In this study, I re-examined the phenomenon using immune mice infected with LCMV, murine cytomegalovirus (MCMV) and vaccinia virus (VACV) in different infection sequences. I confirmed that memory CD8 T cells were

reduced by heterologous infections, and showed that LCMV-specific memory CD4 T cells were also reduced by heterologous infections. Reduction of the memory CD8 T cells is thought to be the result of apoptosis of memory CD8 T cells associated with the peak of type I interferon early during infection. I showed that memory CD4 T cells were similarly driven to apoptosis early during infection; however, Foxp3⁺ CD4⁺ regulatory T cells were relatively resistant to virus infection-induced apoptosis, and were stably maintained during LCMV infection. The stability of Treg cells during viral infections may explain the relatively low incidence of autoimmunity associated with infections.

The history of infections can deviate the course of disease and affect the disease outcome, but this heterologous immunity is not necessarily reciprocal. Previous studies have shown the effects of heterologous immunity during acute infections. In this thesis, I showed that the history of LCMV infection led to higher viral titers during persistent MCMV infection, caused more severe immunopathology at the beginning of infection, and reduced the number of MCMV-specific inflationary memory CD8 T cells after the period of memory inflation. In a different context of infection, the history of LCMV infection can be beneficial. LCMV-immune mice have been shown to have lower viral titers after VACV infection, but VACV-immune mice are not protected during LCMV infection. I found that memory CD8 T cells generated from LCMV and VACV infections were phenotypically different, but the differences could not explain the non-reciprocity of heterologous immunoprotection. By increasing the number of cross-reactive VACV A11R₁₉₈₋₂₀₅-specific memory CD8 T cells, however, I showed that some VACV-immune mice displayed reduced viral titers upon LCMV challenge, suggesting

that the low number of potentially cross-reactive CD8 T cells in VACV-immune mice may be part of the reasons for the non-reciprocity of immunoprotection between LCMV and VACV. Further analysis deduced that both number of potentially cross-reactive memory CD8 T cells and the private specificity of memory CD8 T cell repertoire played a part in determining the outcome of heterologous infections.

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Chapter I. Introduction

The generation of the CD8 T cell response during infections and the establishment of memory CD8 T cells can be influenced by the immunoproteasomes, which dictate the diversity of peptides for presentation to CD8 T cells. The established memory CD8 T cells are stably maintained by homeostatic proliferation, and can affect the immune response to subsequent infections. During heterologous infections, however, the number and frequency of pre-existing memory CD8 T cells to previous infections may be changed. Previous studies have shown that pre-existing memory CD8 T cells may be reduced by heterologous infections, but memory CD4 T cells may be more stable, while the stability of the memory-like regulatory T cells has not been examined.

Heterologous immunity, referring to the immunity that can be developed to an unrelated pathogen during infections, can deviate the course of disease and alter the disease outcome. The effects of heterologous immunity on viral load and immunopathology have been studied during acute infections, but its long-term influence during persistent infections has not been examined. Heterologous immunity is not necessarily reciprocal and the sequence of infections may change the outcome of infections. Prior immunity, which may influence subsequent immune responses, and the biology of subsequent infections are factors that may have contributed to the non-reciprocity of heterologous immunity.

In this thesis, the generation and stability of memory T cells and the influence of history of infections on the outcome of subsequent infections are studied using three unrelated viruses from the families *Arenaviridae*, *Herpesviridae* and *Poxviridae*.

Lymphocytic choriomeningitis virus (LCMV)

Lymphocytic choriomeningitis virus (LCMV) is an enveloped Old World arenavirus with a bi-segmented ambisense single-stranded RNA genome (Bishop & Auperin, 1987; Southern *et al.*, 1987). It enters target cells through the interaction between its glycoprotein GP-1 and the α -dystroglycan on the cell surface (Cao *et al.*, 1998). In mice, LCMV strain Armstrong infects macrophages and some fibroblastic reticular cells in the spleen but not lymphocytes (Matloubian *et al.*, 1993; Mueller *et al.*, 2007). Viral growth is detectable in the spleen by plaque assay beginning at around 5-6 hrs post inoculation, and the progeny viral particles accumulate exponentially (Ehl *et al.*, 1997).

LCMV infections induce a strong type I interferon (IFN $\alpha\beta$) response. LCMV RNA interacts with both melanoma differentiation-associated gene 5 (MDA5) and retinoic acid-inducible gene I (RIG-I) complexes to stimulate type I IFN production through mitochondrial antiviral signaling protein (MAVS) and interferon regulatory factor 7 (IRF7) in conventional dendritic cells (DC) (Zhou *et al.*, 2010; 2012). Through Toll-like receptor 7 (TLR7) and myeloid differentiation primary response gene 88 (MyD88), plasmacytoid DC also contribute to the production of type I IFN in the first 16 hrs post LCMV infection (Wang *et al.*, 2012). The induced type I IFN, which is detectable by 24 hrs and peaks at day 2 post infection (Welsh, 1978), performs pleiotropic functions including stimulating natural killer (NK) cell activities (Mack *et al.*, 2011; Welsh, 1978), supporting clonal expansion of CD8 T cells (Kolumam *et al.*, 2005),

and promoting apoptosis and attrition of CD44^{hi} memory T cells (Bahl *et al.*, 2006; 2010; McNally *et al.*, 2001).

CD8 T cells are essential and sufficient in the control of LCMV infection. LCMV is not cleared from the spleens and livers of mice depleted of CD8 T cells (Moskophidis *et al.*, 1987). Adoptive transfer of 1.5×10^7 cloned LCMV-specific CD8 T cells from LCMV-immune mice can reduce 10^4 - 10^5 pfu LCMV in the spleen to <50 pfu in a day (Byrne & Oldstone, 1984). This CD8 T cell control of LCMV is perforin-dependent. Perforin knockout (KO) mice are unable to control LCMV Armstrong or LCMV-WE infection (Balkow *et al.*, 2001; Kägi *et al.*, 1994; 1995; Walsh *et al.*, 1994), and splenocytes from perforin KO mice cannot transfer protection to naïve recipients either (Kägi *et al.*, 1995). Viral clearance is partially impaired in IFN γ R KO mice and completely blocked in IFN $\alpha\beta$ R KO mice (van den Broek *et al.*, 1995). The containment of infection is a race between replicating LCMV and the activation and proliferation of CD8 T cells, because of the need for contact-dependent killing of LCMV-infected targets. It has been shown that the number of functional cytotoxic CD8 T cells adoptively transferred at the first sign of LCMV WE infection (10 hrs post inoculation) correlates with viral clearance (Ehl *et al.*, 1997). In BALB/c mice, it takes about 10^4 - 10^5 NP118-specific memory CD8 T cells at the time of infection to substantially reduce viral load on day 3 post inoculation of 2×10^5 pfu LCMV Armstrong (Badovinac *et al.*, 2003). That would equate to about 0.1-1% of CD8 T cells, assuming a total of 10^8 cells in the spleen on average and about 10% being CD8 T cells. A more recent study using adoptive transfer of LCMV GP₃₃₋₄₁-specific T cell receptor (TCR)-transgenic P14 CD8 T cells and

peptide-pulsed splenocyte targets into C57BL/6 mice and a mathematical model deduces that 1.3×10^5 memory CD8 T cells are needed for LCMV Armstrong clearance (Ganusov *et al.*, 2011).

The functions of B cells are more apparent during persistent LCMV infections than during acute infections. Depletion of B cells by anti-IgM treatment did not affect the NK and cytotoxic T cell response to acute LCMV infection, nor did it compromise the survival of mice after lethal intracerebral LCMV Armstrong challenge (Cerny *et al.*, 1986). Primary CD8 and CD4 T cell responses and viral clearance were also comparable between μ MT^{-/-} B cell-deficient mice and wild-type (WT) mice after intraperitoneal (i.p.) LCMV Armstrong inoculation (Asano & Ahmed, 1996; Whitmire *et al.*, 2009). However, memory B cells and neutralizing antibody are needed for clearing persistent infection and preventing viral recurrence (Bergthaler *et al.*, 2009; Planz *et al.*, 1997). In neonatal (2-weeks-old) mice, which mount a weak and short-lived CD8 T cell response during low dose LCMV-WE infection, the presence of an elevated level of LCMV NP-specific antibodies becomes critical in viral control (Belnoue *et al.*, 2007). The μ MT^{-/-} B cell-deficient mice also failed to limit viral dissemination during persistent LCMV clone 13 and strain A22-2b infections, and displayed a more severe CD8 T cell exhaustion (Whitmire *et al.*, 2009). Although the activation and expansion of virus-specific CD8 T cells require no B cells, the activated CD8 T cells suffer greater loss during the contraction phase, resulting in lower number of memory CD8 T cells in μ MT^{-/-} B cell-deficient mice (Asano & Ahmed, 1996). Memory CD4 T cells are not generated in μ MT^{-/-} B cell-deficient mice (Whitmire *et al.*, 2009), and the compromised CD4 T cell

compartment contributes to the loss of CD8 T cells during persistent LCMV infections (Matloubian *et al.*, 1994).

Vaccinia virus (VACV)

Vaccinia virus (VACV) is an orthopoxvirus with an envelope and a double-stranded DNA genome. The coding regions with mixed reading directions (designated L for leftward reading and R for rightward reading) are organized with no gaps in between (Goebel *et al.*, 1990). The origin of VACV is not known, but it was used as a vaccine, which led to the eradication of smallpox among the human population in the late 1970's (Buller & Palumbo, 1991). VACV strain Western Reserve (WR) infects human CD14+ monocytes, B cells and *in vitro* activated T cells, but not quiescent T cells or CD56+ NK cells (Chahroudi *et al.*, 2005; Sánchez-Puig *et al.*, 2004). In mice, DC and some macrophages are the primary targets of infection among the hematopoietic cells (Liu *et al.*, 2008).

VACV infection elicits a broad spectrum of cellular immune responses with overlapping functions in mice. In the first few days of infection $\gamma\delta$ T cells respond by producing IFN γ (Selin *et al.*, 2001). Thereafter, CD4 and CD8 T cells join the efforts by secreting IFN γ (Xu *et al.*, 2004). CD4 T cells also shape the humoral response, and CD4-depleted mice or major histocompatibility complex (MHC) class II KO mice fail to generate a VACV-specific IgG response (Xu *et al.*, 2004). The control of VACV infection is not dependent on perforin or Fas/FasL pathways of cytotoxicity (Kägi *et al.*, 1995). In fact, Fas-deficient *lpr* mice clear VACV better and survive high dose VACV

infection probably because of the greater number of IFN γ -producing NK cells and activated T cells in these mice prior to infection (Seedhom *et al.*, 2012).

IFNs are important in the immune defense against VACV. IFN $\alpha\beta$ R or IFN γ R KO mice are unable to control VACV infection (van den Broek *et al.*, 1995). Intranasal administration of IFN α or IFN γ before and after VACV inoculation can rescue BALB/c mice from the lethal intranasal infection (Liu *et al.*, 2004). IFN γ may exert its antiviral functions through macrophages. It has been shown in the mouse leukemia monocyte macrophage cell line RAW 264.7 that IFN γ stimulates the expression of nitric oxide synthase (iNOS) and blocks VACV protein synthesis and DNA replication (Harris *et al.*, 1995; Karupiah *et al.*, 1993). Therefore, memory T cells with IFN γ -secreting capability are protective during subsequent challenges. Heterologous immunity against VACV is mediated by IFN γ -producing CD8 and CD4 T cells (Selin *et al.*, 1998), and the frequency of IFN γ -producing memory CD8 T cells generated by peptide-immunization correlates positively with the survival of C57BL/6 mice from lethal intranasal VACV challenge (Moutaftsi *et al.*, 2009; Salek-Ardakani *et al.*, 2008).

VACV is sensitive to antibody neutralization. The transfer of immune serum prior to VACV infection is sufficiently protective, and immunized mice are protected from homologous challenge even after depletion of CD8 or CD4 T cells (Belyakov *et al.*, 2003).

Murine cytomegalovirus (MCMV) and memory inflation

Murine cytomegalovirus (MCMV) is a β -herpesvirus that causes persistent infection in mice. Macrophages and mesothelial cells lining the peritoneum in case of i.p. infection are the first targets of infection (Hsu *et al.*, 2009; Stoddart *et al.*, 1994). Infection spreads to the spleen and liver through the circulation within hours (Hsu *et al.*, 2009). Infectious virus and viral DNA can be found in spleen (Chong *et al.*, 1981), and in circulating leukocytes of susceptible mouse strains (BALB/c and C57BL/6) (Bale & O'Neil, 1989) within 2-3 days post inoculation. Dissemination through mononuclear phagocytes to other organs like kidney, lung and salivary glands follows (Hsu *et al.*, 2009; Stoddart *et al.*, 1994). Productive infection is cleared quickly except from the salivary glands (Reddehase *et al.*, 1994), where viral burden peaks on around day 21 and lasts for weeks. Latency develops after about 2-3 months post infection in adult mice (Henson & Strano, 1972; Reddehase *et al.*, 1994) and viral DNA can be detected in macrophages and in endothelial cells in various organs (Koffron *et al.*, 1998; Seckert *et al.*, 2009). Reactivation occurs spontaneously at low frequency in the salivary gland, and is inducible in other organs (e.g. lung and spleen) by immunosuppression (Balthesen *et al.*, 1993; Jordan *et al.*, 1977; Reddehase *et al.*, 1994) or i.p. injection of lipopolysaccharide or pro-inflammatory cytokines, tumor necrosis factor (TNF) or interleukin (IL)-1 β (Cook *et al.*, 2006).

The virulence of MCMV is influenced by how the virus has been passaged. A single passage in cell cultures or in the mouse salivary glands can quickly change the virulence of MCMV (Osborn & Walker, 1971) and its resistance to antibody neutralization *in vitro* (Chong *et al.*, 1981). Virulent MCMV, that is lethal to suckling

mice, can only be produced from the salivary glands of infected mice but not from inoculated salivary gland cultures (Jordan & Takagi, 1983; Selgrade *et al.*, 1981). The virus-specific non-neutralizing antibodies-decorated membrane of the *in vivo*-generated virus might have increased the resistance to neutralization (Chong *et al.*, 1981). The timing of harvest also affects virulence, and the viruses collected from murine salivary glands at 2-3 weeks post infection are more virulent than viruses collected at 5-6 weeks post inoculation (Selgrade *et al.*, 1981).

Host defense against MCMV infection employs a combination of innate and adaptive immune strategies with some functional redundancies, and yet certain arms of the immune response are more important in certain organs at a particular stage of disease. The NK cell response dominates early viral control of MCMV in the spleen and liver through perforin-dependent and IFN γ /NOS-dependent mechanisms, respectively, until the T cell response develops (Tay & Welsh, 1997; Welsh *et al.*, 1991). CD4 T cells control MCMV in the salivary gland by suppressing viral replication in radio-resistant acinar glandular epithelial cells through secretion of IFN γ (Walton *et al.*, 2008; 2011a). Mice can survive infection without NK or CD4 T cells but the depletion of either population increases the viral burden in the salivary glands (Podlech *et al.*, 1998; Polić *et al.*, 1996; Welsh *et al.*, 1991). The absence of a humoral response does not affect the initiation or clearance of MCMV infection in any organ but invigorates the spread of virus after reactivation by gamma-irradiation (Jonjić *et al.*, 1994), suggesting a role for B cells in viral control during recurrence. Adoptive transfer of memory B cells provides prophylactic and therapeutic benefits in the immunodeficient RAG-1 KO mice against

lethal MCMV infection (Klenovsek *et al.*, 2007), and the administration of anti-MCMV serum can also limit the spread of virus and minimize pathology in the neonates (Cekinović *et al.*, 2008). Some T cell function is necessary for survival, but whether or not the CD8 T cell response is essential has been controversial. Viral control without CD8 T cells in the long-term depleted mice and in $\beta 2m$ KO mice is as efficient as the undepleted and WT control respectively (Jonjić *et al.*, 1990; Polić *et al.*, 1996), suggesting a redundant role of CD8 T cells in viral control. However, the absence of CD8 T cells increases the vulnerability of mice to lethal infection with a high dose of more virulent MCMV salivary gland isolate (Polić *et al.*, 1996), and depleting CD8 T cells in MCMV-infected bone marrow transplant recipient mice can be fatal because of multi-organ dissemination of replicating virus (Podlech *et al.*, 1998). Functional MCMV-specific effector and memory CD8 T cells are effective in clearing virus from the spleen, lungs, liver, brain and small intestine (Podlech *et al.*, 1998). At least three immunoevasins (m04, m06 and m152) target the MHC class I presentation to CD8 T cells (Reddehase, 2002). These findings support the significance of CD8 T cells in host defense against MCMV.

MCMV-specific memory CD8 T cells exist in different expansion and contraction patterns. The non-inflatory memory, also referred to as conventional memory, expands and contracts during acute infection and then remains stable at low numbers. Examples are M45₉₈₅₋₉₉₃- and M57₈₁₆₋₈₂₄-specific CD8 T cells, whose numbers peak on around day 7 post infection. CD8 memory T cells specific for m139₄₁₉₋₄₂₆, M38₃₁₆₋₃₂₃, IE3₄₁₆₋₄₂₃ in C57BL/6 mice and for pp89₁₆₈₋₁₇₆ and m164₂₅₇₋₂₆₅ in BALB/c

mice are examples of the inflationary memory (Munks *et al.*, 2006a; Sierro *et al.*, 2005). All except IE3₄₁₆₋₄₂₃-specific CD8 T cells are readily detectable during the acute phase of infection. The CD8 T cells specific for m139₄₁₉₋₄₂₆ in C57BL/6 mice and for pp89₁₆₈₋₁₇₆ and m164₂₅₇₋₂₆₅ in BALB/c mice peak at around day 7 post infection, contract briefly, and increase in number again until reaching a peak at around day 100-120. M38₃₁₆₋₃₂₃-specific CD8 T cell number continues to increase steadily beyond day 120. IE3₄₁₆₋₄₂₃-specific CD8 T cells are undetectable until 4 weeks post infection. All inflationary memory T cells increase in number between 6-12 weeks post MCMV infection (Munks *et al.*, 2006a; Sierro *et al.*, 2005; Snyder *et al.*, 2008). Memory T cell population can be segregated phenotypically into the effector memory T cells, which are dispersed in the periphery and perform immediate effector functions, and the central memory T cells, which express homing receptors to secondary lymphoid organs, CCR7 and CD62L, proliferate readily and differentiate into effector cells upon re-stimulation (Sallusto *et al.*, 2004). Over time the non-inflationary memory T cells take on a more central memory phenotype and become CD62L^{hi} CD27^{hi} CD122^{hi}, while the inflationary memory T cells resemble effector memory with a phenotype of CD62L^{lo} CD27^{lo} CD122^{lo} KLRG1^{hi} (Sierro *et al.*, 2005; Snyder *et al.*, 2008). Inflationary memory CD8 T cells accumulate in the spleen and lungs but not in lymph nodes, where virus does not persist (Torti *et al.*, 2011a).

The generation of MCMV-specific CD8 memory T cells involves both direct- and cross-presentation of MCMV-specific peptide epitopes. The CD8 T cell response to MCMV is primarily primed by cross-presenting CD103⁺ CD8 α ⁺ CD11c⁺ DC (Snyder *et*

al., 2010; Torti *et al.*, 2011b). The immunoproteasome subunit low-molecular mass polypeptide 7 (LMP7) is needed for both the acute response and memory generation (Hutchinson *et al.*, 2011). MHC class I-presented antigens on non-hematopoietic cells are responsible for stimulating memory inflation (Seckert *et al.*, 2011; Torti *et al.*, 2011a). Re-activation of the viral genes in infected cells occurs sporadically throughout life and is thought to be the source of antigen that triggers T cells to expand, leading to memory inflation (Seckert *et al.*, 2012). Although memory inflation begins as early as 3 weeks post infection – several weeks before the beginning of latency as defined by the absence of infectious virus in any organ, neither the suppression of viral replication or the removal of the salivary glands reduce memory inflation (Loewendorf *et al.*, 2011; Snyder *et al.*, 2011; Walton *et al.*, 2011b), suggesting that the antigen for continuous stimulation of the inflationary memory T cells does not come from active viral replication during viral persistence in the salivary glands. The comparable proliferation kinetics of inflationary memory CD8 T cells in WT versus CD4 T cell-deficient (CD4 KO and MHC II KO) mice (Snyder *et al.*, 2009) also suggests that the duration of virus persistence may not dictate how long CD8 T cell memory inflates. However, MCMV viral load may determine the size of memory inflation. BALB/c mice retain a higher MCMV viral load than C57BL/6 mice (Vliegen *et al.*, 2003), and the inflation of memory specific to pp89 is also more dramatic – up to 20% of CD8 T cells at one year post infection (Karrer *et al.*, 2003).

Heterologous Immunity

The history of infection(s) can deviate the course of disease and influence the outcome of subsequent infections. The effects may be beneficial and will reduce viral load and increase survival of infected animals, but they may also be harmful and prevent efficient viral clearance and/or cause immunopathology. Sometimes, however, improved viral clearance may be accompanied by more severe immunopathology, as in the case of VACV infection in LCMV-immune mice. Infection of LCMV-immune mice with VACV results in a reduction of about 1.5 log pfu/ml of VACV titer 3-4 days after infection compared to non-immune mice (Chen *et al.*, 2001a; Selin *et al.*, 1998), and the LCMV-immune mice are also protected from death after intranasal VACV infection (Chen *et al.*, 2001a). However, the history of LCMV infection or the adoptive transfer of LCMV-immune splenocytes can cause more severe inflammation and necrosis of the abdominal fat pads in intraperitoneally VACV-infected mice (Nie *et al.*, 2010; Selin *et al.*, 1998). The effects of heterologous immunity may be long lasting during persistent infections. Using LCMV-immune mice, the effects of the history of infection were studied during MCMV persistent infection and will be discussed in Chapter VI.

Memory CD8 T cells can mediate both immunoprotection and immunopathology through IFN γ , but the two phenomena are not necessarily linked. LCMV-specific CD8 T cells are protective during VACV challenge. CD8 T cell depletion abolishes the protective effect of adoptively transferred LCMV-immune splenocytes in VACV-infected hosts (Selin *et al.*, 1998), and the transfer of cross-reactive T cell lines generated from LCMV-immune mice can reduce viral burden of VACV-infected hosts (Cornberg *et al.*, 2010), but IFN γ R KO LCMV-immune mice no longer clear virus more efficiently or

develop immunopathology in visceral fat pads upon VACV challenge (Selin *et al.*, 1998). On the contrary, a history of influenza infection enhances viral load and causes pathology in the lung during LCMV infection (Chen *et al.*, 2003; Wlodarczyk *et al.*, 2013). Depleting memory CD8 T cells from influenza-immune mice, tolerizing the influenza-immune mice with cross-reactive peptides, or blocking IFN γ signals with monoclonal antibody reduces lung pathology upon LCMV infection but has no influence on LCMV clearance (Wlodarczyk *et al.*, 2013).

Similar to CD8 T cells, CD4 T cells can be involved in heterologous immunity. VACV infection of the antibiotic-treated *Mycobacterium bovis* bacillus Calmette-Guerin (BCG)-immune mice results in a 1-3 log reduction of viral titer in 1-3 days post infection, and the protection is abrogated by depleting CD4 T cells prior to infection, or by neutralizing the IFN γ secreted by CD4 T cells (Mathurin *et al.*, 2009).

CD25⁺ CD4⁺ regulatory T (Treg) cells also contribute to heterologous immunity. A recent study on influenza-immune mice revealed elevated numbers of Treg cells in the lung, mediastinal lymph nodes and the spleen six weeks after influenza infection, and upon intranasal LCMV infection, the accumulation of Treg cells in the lung and the secondary lymphoid organs correlates with more severe CD8 T cell-mediated lung pathology; this is reversible by depleting Treg cells from influenza-immune mice using an anti-CD25 antibody prior to LCMV challenge (Kraft *et al.*, 2013). It was deduced that the elevated number of Treg cells in the influenza-immune mice might have prevented the partial exhaustion of LCMV-specific CD8 T cells, and supported the migration of CD8 T cells out of the lymph nodes into the lung. Consequently, the higher number of

activated CD8 T cells in the presence of high viral load led to more severe lung pathology.

The effect of the history of infection(s) is not necessarily reciprocal, and the sequence of infections may determine the disease outcome. A history of infection with LCMV, MCMV, Pichinde virus (PV), BCG or influenza A virus provides a certain level of heterologous protective immunity against VACV and reduced viral titer by one to two log pfu early (day 3-4) after infection (Chen *et al.*, 2003; Mathurin *et al.*, 2009; Selin *et al.*, 1998). However, a prior history of VACV infection does not protect against subsequent challenge with LCMV, MCMV or PV (Selin *et al.*, 1998). A history of LCMV infection protects against PV challenge to a greater extent than the history of PV infection does to LCMV infection (Selin *et al.*, 1998). Differences in the biology of the infections and the host responses may have contributed to the non-reciprocal nature of heterologous immunity. The specific situation between LCMV and VACV was studied and will be discussed in Chapter VII.

Cross-reactivity between viruses

T cells recognizing one pathogen may respond to epitopes from another unrelated pathogen. Cross-reactive T cells can bind to the corresponding tetramers simultaneously, albeit with varying avidities. LCMV NP₂₀₅₋₂₁₂, GP₃₄₋₄₁, GP₁₁₈₋₁₂₅ and VACV A11R₁₉₈₋₂₀₅ are cross-reactive between LCMV and VACV (Cornberg *et al.*, 2010; Kim *et al.*, 2005), and cross-reactive CD8 T cell lines can co-stain with both VACV A11R₁₉₈₋₂₀₅ tetramer and LCMV GP₃₄₋₄₁ or GP₁₁₈₋₁₂₅ tetramer (Cornberg *et al.*, 2010). NP₂₀₅₋₂₁₂ is cross-

reactive between LCMV and PV, and the effector CD8 T cells from LCMV-infected mice also co-stain with LCMV NP₂₀₅₋₂₁₂ and PV NP₂₀₅₋₂₁₂ tetramers (Cornberg *et al.*, 2006). A recent study demonstrated the dual specificity of peptide-MHC binding in a single CD8 T cell using bi-specific heterodimers, which are chemically linked dimers constructed between A11R₁₉₈₋₂₀₅-H-2K^b monomers and GP₃₄₋₄₁-H-2K^b monomers (Shen *et al.*, 2010). The monomers by themselves, or the dimers between the monomer and a non-specific monomer do not associate TCR with sufficient avidity, but the heterodimer binds with similar strength as the GP₃₄₋₄₁-H-2K^b homodimer does, suggesting the existence of TCR with dual binding specificities or the co-existence of TCRs with different specificities on a single T cell surface. Incomplete allelic exclusion can give rise to T cells expressing two functional V α , but in mice the frequency of dual chain T cells in circulation seems rather low (Alam & Gascoigne, 1998; Heath *et al.*, 1995). The degeneracy of TCR recognition of peptide-MHC complexes may allow the same TCR to recognize more than one specific peptide sequence. The complementarity determining region 3 (CDR3) loops on the TCR are flexible, enabling the TCR to interact with epitopes of different amino acid sequences (Reiser *et al.*, 2003). Substitutions on the peptide that achieve similar TCR-interacting surfaces are equally recognized (Sandalova *et al.*, 2005). Besides, because of the way peptides are presented in the MHC binding groove, only a few residues are actually exposed and interact with the TCRs (Fremont *et al.*, 1992; Rudolph & Wilson, 2002; Zhang *et al.*, 1992). Certain substitutions of the anchoring residues are tolerated as long as they do not disrupt the binding to the MHC molecule (Reiser *et al.*, 2003). Therefore, some peptides with little or no sequence

homology may be recognized by the same TCR. In fact, cross-reactive epitope pairs do not always share sequence homology (Reiser *et al.*, 2003; Welsh *et al.*, 2010).

T cell cross-reactivity may lead to alteration of the immunodominance hierarchy and the V β repertoire of an immune response. During heterologous infection, pre-existing memory T cells recognizing their cross-reactive epitope will clonally expand, resulting in alteration of the immunodominance hierarchy of the T cell response and the hierarchy of memory T cells (Brehm *et al.*, 2002; Kim *et al.*, 2002; Selin *et al.*, 2006). During the expansion, alteration and narrowing of the TCR V β repertoire is possible, because only a subset of the epitope-specific T cells may respond to the cross-reactive epitope, and a limited number of clonotypes will dominate the majority of the TCRs in the double immune mice (Cornberg *et al.*, 2006).

Private specificity of T cell response

The distinct repertoires of TCR constitute the private specificity of the cross-reactive response during heterologous infections. During T cell development in the thymus, the variable (V)-diversity (D)-joining (J) recombination of the TCR β chain and the V-J joining of TCR α chain give rise to the diversity of TCR. Additionally, the terminal deoxynucleotidyl transferase (TdT) further creates a distinct repertoire of TCR for each individual mouse by randomly incorporating extra nucleotides to the junction of the rearranged DNA segments. The sequence in the V segment forms the CDR1 and CDR2 regions while the rearranged V-D-J (or V-J) segments form the highly variable CDR3 loops in the center of the TCR. In LCMV-immune mice, although some T cells

specific for NP₂₀₅₋₂₁₂, GP₃₄₋₄₁ and GP₁₁₈₋₁₂₅ are cross-reactive with VACV A11R₁₉₈₋₂₀₅, VACV infection preferentially expands only one or two (but not all three) of the cross-reactive epitope-specific T cell populations within an individual mouse, and the pattern of cross-reactive T cell expansion differs between mice (Kim *et al.*, 2005). In PV-infected LCMV-immune mice, the V β repertoire of the NP₂₀₅₋₂₁₂-specific CD8 T cells may become more oligoclonal because only certain clonotypes of the TCRs may interact with the cross-reactive peptide-MHC complex with sufficient affinity to trigger proliferation of the memory CD8 T cells, and the pattern of skewing varies between mice, with distinct V β dominating in different mice (Cornberg *et al.*, 2006).

The phenomenon of private specificity gives rise to differential disease outcome among mice of the same genetic background. Protection from subsequent infections can be influenced by the private specificity of the cross-reactive T cell repertoire of the immune host. VACV A11R₁₉₈₋₂₀₅-specific T cell lines generated from LCMV-immune mice are protective during VACV infections but the level of protection varies, reflecting the TCR repertoire of the specific T cell line. These TCR repertoires influence the ability of the memory T cell population to protect against heterologous VACV infection (Cornberg *et al.*, 2010). The private specificity of the T cell repertoire of the immune donor also governs the occurrence of immune pathology, and in VACV-infected recipients of LCMV-immune splenocytes, a more focused response towards GP₁₁₈₋₁₂₅ seemed to associate with more severe necrosis in the visceral fat pads (Nie *et al.*, 2010).

Maintenance of memory CD8 T cells

The number and frequency of memory CD8 T cells is generally stably maintained but memory CD4 T cell number may decline over time. The stability of CD8 T cell memory was examined in LCMV-, PV- and VACV-immune mice using irradiated, virus-infected PEC as feeders in limiting dilution CTL assays and found to be stable over time (Selin *et al.*, 1996). The observation was later confirmed in LCMV-immune mice using virus-specific peptides in intracellular cytokine assays (Homann *et al.*, 2001; Selin *et al.*, 1999). In the same study, the number of memory CD4 T cells was found to gradually decline over time (Homann *et al.*, 2001).

Memory CD8 T cells are maintained through homeostatic proliferation supported by IL-7 and IL-15 (Burkett *et al.*, 2003; Prlic *et al.*, 2002; Schluns *et al.*, 2000), but MHC engagement is not required for their maintenance (Murali-Krishna *et al.*, 1999; Tan *et al.*, 2002). IL-7 is produced in the thymus, bone marrow, spleen, lymph nodes, intestinal epithelium and skin epidermal basement membrane (Hara *et al.*, 2012; Link *et al.*, 2007; Mazzucchelli *et al.*, 2009; Wagner *et al.*, 1999). Both DC and macrophages are responsible for transpresenting IL-15 to CD8 T cells (Frasca *et al.*, 2010; Mortier *et al.*, 2009). Although both IL-7 or IL-15 stimulation can support homeostatic proliferation of CD44^{hi} CD8 T cells in irradiated hosts (Kieper *et al.*, 2002; Schluns *et al.*, 2000; Tan *et al.*, 2002), and homeostatic proliferation is impaired in IL-15 KO and IL-15Ra KO mice (Schluns *et al.*, 2002), IL-15 does not seem to be required for survival of non-dividing memory CD8 T cells because the number of undivided adoptively transferred memory CD8 T cells were not reduced in IL-15 KO hosts after 30 days (Becker *et al.*, 2002). Both IL-7 and IL-15 signal through STAT5. Constitutively activated STAT5 boosts

proliferation of memory CD8 T cells (Hand *et al.*, 2010). Myeloid cells of mixed DC phenotypes may also enhance the survival of memory CD8 T cells, especially those of CD62L^{hi} phenotype, through CD70 signaling to CD27-expressing CD8 T cells (Frasca *et al.*, 2010).

Viral infections can cause a reduction of memory CD8 T cells (Brehm *et al.*, 2002; Kim & Welsh, 2004; Liu *et al.*, 2003; Selin *et al.*, 1996; 1999; Varga *et al.*, 2001). The peak of virus-induced type I IFN early during LCMV infection coincides with the reduction of CD44^{hi} memory CD8 T cells and is thought to be the cause of memory attrition and early apoptosis of CD44^{hi} CD8 T cells. Experiments have shown that the direct injection of IFN $\alpha\beta$ or IFN $\alpha\beta$ -inducer poly(I:C) resulted in the loss of memory CD8 T cells and CD44^{hi} CD8 T cell apoptosis, but IFN $\alpha\beta$ R KO mice were resistant to the reduction of CD8 T cells at day 3 post LCMV infection (Bahl *et al.*, 2006; Jiang *et al.*, 2005; McNally *et al.*, 2001). Infection-induced lymphopenia also contributes to the reduction of pre-existing memory CD8 T cells because the pathogen-specific memory T cells proliferates at a lower rate in the absence of their cognate antigens and are outcompeted by other dividing T cells (Peacock *et al.*, 2003).

Bacterial or protozoal infections can also disturb the stability of memory T cell populations. Pre-existing *Listeria monocytogenes* (LM)-specific memory CD8 T cells increase during heterologous chronic *Leishmania donovani* infection, and memory CD8 T cells of central memory phenotype are preferentially accumulated (Polley *et al.*, 2005). These LM-specific CD8 T cells do not proliferate upon stimulation by *Leishmania*-infected DC and are presumably not accumulated by cross-reactivity. On the other hand,

infections with BCG or *Salmonella typhimurium*, which can also cause chronic infection, erode pre-existing LM-specific memory CD8 and CD4 T cells through an IFN γ -dependent mechanism (Dudani *et al.*, 2008; Smith *et al.*, 2002). Moreover, exposure to bacterial superantigens can also result in loss of memory CD8 T cells. *Staphylococcal* enterotoxin B (SEB) interacts with TCR V β and causes activation and proliferation of memory CD8 T cells (Coppola & Blackman, 1997), but then selectively destroys V β 8.3+ memory CD8 T cells through activation-induced cell death (Huang *et al.*, 2002).

Regulatory T (Treg) cells and their maintenance

Treg cells constitute a heterogeneous T cell population that plays an important role in maintaining self-tolerance and limiting an overactive immune response. Immunosuppressive “natural” Treg cells develop in the thymus and are marked by the expression of CD4 and a Forkhead/winged-helix family member, forkhead box P3 (Foxp3; Scurfin), which is an activator and a repressor of transcriptional control (Zheng *et al.*, 2007). Foxp3 forms a 400-2000 kDa complex, the “Foxp3 interactome” with many partners, including some transcription factors, histone methyl transferases, and chromatin remodeling complexes (Rudra *et al.*, 2012), and it functions in a transcription control network with other transcription factors (Fu *et al.*, 2012) to generate signature phenotypes in Treg cells (Williams & Rudensky, 2007). Scurfy mice have a 2-base pair insertion in their exon 8 of the X-linked Foxp3 gene (Brunkow *et al.*, 2001) and develop a lymphoproliferative disorder and perivascular infiltration of hematopoietic cells in multiple peripheral organs (Godfrey *et al.*, 1991). Experimentally depleting the Foxp3-

expressing population in mice can lead to autoimmune disorders (Feuerer *et al.*, 2009; Kim *et al.*, 2006b; Lahl *et al.*, 2007).

During infections, Treg cells can mitigate the pathology incurred by immune response and prolong survival of the infected mice. Weight loss and accumulation of monocytes and macrophages in the lungs after influenza PR8 infection are reduced after adoptive transfer of CD25⁺ CD4⁺ Treg cells, which modulate the timing of production of chemokine CCL8 in the lungs (Antunes & Kassiotis, 2010). A later study also shows that transferred virus-reactive Treg cells can down-regulate the accrual of effector CD4 and CD8 T cells in the lungs and modulate systemic cytokine responses after PR8 infection (Bedoya *et al.*, 2013). In mice infected ocularly with herpes simplex virus (HSV)-1, Foxp3⁺ Treg cells reduce the severity of corneal stromal keratitis lesions by suppressing the production of inflammatory cytokines and chemokines and limiting the infiltration of CD4 T cells, neutrophils and macrophages to the cornea (Veiga-Parga *et al.*, 2012). In some situations Treg cells control disease severity through enhancement of effector responses to infections and efficient viral clearance. Treg cells promote migration of effector CD4 T cells, NK cells, CD11b⁺ DC and plasmacytoid DC from the draining lymph nodes to the site of infection through modulation of chemokine production in both locations early during genital HSV-2 infection, and mice depleted of Foxp3⁺ Treg cells have higher viral titers in the reproductive organ and the spinal cord and suffer a more severe disease earlier (Lund *et al.*, 2008). Along the same lines, Treg cells support the migration of virus-specific CD8 T cells to the lungs during respiratory syncytial virus infection, and CD25⁺ Treg cell-depleted mice experience delayed viral clearance and

more severe weight loss and airway resistance at 1 week after infection (Fulton *et al.*, 2010).

Foxp3⁺ Treg cells require IL-2 signaling for survival and non-lymphopenic self-renewal (Fontenot *et al.*, 2005; Setoguchi *et al.*, 2005). They themselves do not produce IL-2 (Su *et al.*, 2004; Takahashi *et al.*, 1998), because Foxp3 displaces AP-1 and suppresses the expression of IL-2 through cooperative binding with NFAT on the *Il2* promoter (Wu *et al.*, 2006) with the help of Helios (Baine *et al.*, 2013). Therefore, Treg cell maintenance depends on IL-2 secreted by other cells, and treating mice with high dose anti-IL-2 antibody reduces the number of Foxp3⁺ CD4⁺ Treg cells, especially in the periphery, and initiates autoimmune disorders (Setoguchi *et al.*, 2005). IL-2 increases Foxp3 expression per Treg cell and causes expansion of Treg cell populations (Fontenot *et al.*, 2005; Murawski *et al.*, 2006; Zorn *et al.*, 2006). The importance of IL-2 to Treg cell survival is evident in IL-2 KO mice, where adoptively transferred WT Treg cells do not survive (Malek *et al.*, 2002). Both CD25 KO and IL-2 KO mice can succumb to lymphoproliferative disorders, albeit relatively delayed compared to Foxp3 KO mice (Fontenot *et al.*, 2005).

IL-2 also influences Treg cell function. CD4 T cells from IL-2 KO mice, but not from CD25 KO mice, perform Treg cell suppressive functions and prevent the development of autoimmune conditions in IL-2-normal disease-prone recipient mice (Furtado *et al.*, 2002), suggesting a role of IL-2 in the function of Treg cells in the periphery. The Foxp3⁺ Treg cell population rescued by blocking apoptosis in B-cell lymphoma (Bcl)-2-interacting mediator of cell death (Bim) and IL-2 double KO mice

were not sufficient in preventing autoimmunity in mice (Barron *et al.*, 2010), suggesting a role of IL-2 beyond maintaining survival of Treg cells. However, IL-2 is not absolutely required in the thymic development of Foxp3⁺ Treg cells, and Foxp3⁺ CD4⁺ Treg cells from both IL-2 KO and CD25 KO mice are capable of suppressing proliferation of WT conventional T cells *in vitro* (Fontenot *et al.*, 2005). IL-2 may have stimulated proliferation of Treg cells, and contributed to the enhanced suppressive functions of the IL-2 KO T cell population through increased Treg cell number. The capability of Treg cells to suppress may be a combination of both total Treg cell number and per cell suppressive capability.

Although Treg cells may not survive as well by themselves in purified cultures because of their dependence on common γ chain cytokines secreted from other cells (Kaminitz *et al.*, 2010; Pandiyan & Lenardo, 2008), they are more resistant to apoptosis than other cells *in vivo*. After whole body gamma-irradiation, CD8 and CD4 T cells were much reduced, but CD25^{hi} CD4 Treg cells were proportionally increased (Qu *et al.*, 2010). Human Treg cells have been found more resistant to apoptosis induced by irradiation or topoisomerase inhibitors than non-Treg CD4 T cells (Winzler *et al.*, 2011). Both murine and human Treg cells are resistant to activation-induced cell death (Banz *et al.*, 2002; Fritzsching *et al.*, 2005). In Chapter V, the relative resistance of Treg cells to apoptosis during LCMV infection will be discussed. It is unclear why Treg cells may be more resistant to cell death in some situations. The study in mice correlated higher Bcl-2 expression in CD25^{hi} CD4 Treg cells to their resistance to irradiation-induced cell death (Qu *et al.*, 2010). However, Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) expressions

were found comparable in human Treg vs effector T cells in a different study (Winzler *et al.*, 2011).

The immunoproteasome and immune response

The peptides presented by antigen-presenting cells can influence the diversity of T cell response. Professional antigen-presenting cells like DC and macrophages express both constitutive proteasomes and immunoproteasomes (Nil *et al.*, 2004). The constitutive 26S proteasome consists of a 20S core and a 19S regulatory complex (PA700) at one or both ends of the 20S core (Coux *et al.*, 1996). The 19S regulatory complex is involved in degrading ubiquitinated protein substrates in an ATP-dependent manner (Kish-Trier & Hill, 2013). The 20S core contains 28 subunits arranged in a cylindrical structure of four concentric rings, each with seven different subunits. The 14 α -type subunits form the two outer rings, and the 14 β -type subunits constitute the two rings in the middle. The β -type subunits δ (β 1), MB1 (β 5) and MC14 (β 2) are catalytically active. The immunoproteasome shares the same basic format, except for the replacement of the 19S regulatory complex by the 11S activator (PA28 complex) on one end of the 20S core and the three catalytic β -type subunits by the inducible homologues – the LMP2 (β 1i), LMP7 (β 5i), and multicatalytic endopeptidase complex-like-1 (MECL-1; β 2i).

IFN γ stimulation can induce the expression of the alternative β -type subunits, which equip the immunoproteasome with modulated endopeptidase activities (Aki *et al.*, 1994; Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993; Groettrup *et al.*, 1996). Early studies

with LMP2 KO mice suggested that LMP2 increased chymotrypsin- and trypsin-like activities and decreased peptidyl glutamyl peptide hydrolase- or caspase-like activity (Van Kaer *et al.*, 1994), but since LMP2 and MECL-1 were coordinately recruited to the immunoproteasome (Groettrup *et al.*, 1997), MECL-1 might have partaken in the changes. A later study using an inactive MECL-1 T1A construct to displace MC14 in the transfectant fibroblasts determined that MECL-1 or MC14 alone were responsible for the trypsin-like activity of the proteasome (Salzmann *et al.*, 1999). This was later confirmed in the MECL-1 KO mice, and the observation that only caspase-like activity was elevated in the 20S proteasomes isolated from MECL-1 KO mice, but the trypsin- and chymotrypsin-like activities were not different from WT mice, suggested that both MECL-1 and MC14 might have similar trypsin-like activity (Basler *et al.*, 2006). On the other hand, the comparison of chromatography-purified proteasomes from WT and LMP7 KO mice revealed that LMP7 participated in the chymotrypsin-like activity of the 20S proteasomes (Stohwasser *et al.*, 1996).

With the presence of LMP2, LMP7 and MECL-1, the immunoproteasomes produce more hydrophobic and basic substrates suitable for binding to the MHC class I molecules (Driscoll *et al.*, 1993; Falk *et al.*, 1991; Gaczynska *et al.*, 1993). In fact, the immunoproteasome generates quite a different repertoire of peptides, as demonstrated by comparing peptide pools generated from the *in vitro* digestion of enolase by LMP2/LMP7 double-deficient proteasomes and immunoproteasomes isolated from transformed B cells (Toes *et al.*, 2001), and by the analysis of acid-eluted peptides from the bone marrow-derived dendritic cells (BMDC) of WT and LMP7/MECL-1 double KO mice (de Verteuil

et al., 2010). By modulating the cut site preference, the immunoproteasomes may generate peptides that are more easily transported by transporter associated with antigen processing (TAP) across the endoplasmic reticulum (ER) membrane, thereby increasing the representation of certain epitopes such as LCMV NP₁₁₈₋₁₂₆ in the peptide pool (Schwarz *et al.*, 2000). In contrast, the immunoproteasome may destroy some epitopes by favoring a cut site within the epitope, such as LCMV GP₂₇₆₋₂₈₆ by LMP2, and thwart their presentation (Basler *et al.*, 2004). Besides their catalytic roles, the immunoproteasome subunits may protect some epitopes by their structural contribution to the proteasomes. Using LMP2-specific enzyme inhibitors and the inactive LMP2 T1A construct, the structural presence of LMP2, and not its enzymatic activity, was found to protect the male-specific antigen UTY₂₄₆₋₂₅₄ (Basler *et al.*, 2012). Similarly, influenza M1₅₈₋₆₆ was found to be protected by the structural presence of and not by the enzymatic activity of LMP7 from being destroyed by the constitutive β 5 subunit (Basler *et al.*, 2012; Gileadi *et al.*, 1999).

The changes in the peptide pool caused by the immunoproteasomes may influence the immunodominance hierarchy of CD8 T cell responses to pathogens. The specific responses to LCMV GP₂₇₆₋₂₈₆ and NP₂₀₅₋₂₁₂ were lower in MECL-1 KO mice at day 8 post infection (Basler *et al.*, 2006). At day 7 post MCMV infection, LMP7 KO mice also displayed a much reduced CD8 T cell response, especially towards the non-inflationary epitopes (Hutchinson *et al.*, 2011).

The overall increase in peptide output by the immunoproteasome can affect the density of MHC class I molecules on the surface of antigen-presenting cells. The LMP7

KO reduced the presence of MHC class I molecules on the cell surface (Fehling *et al.*, 1994). Reduced surface expression of MHC class I molecules was also observed on the BMDC of LMP7/MECL-1 double KO mice, and in that study the lower abundance of peptides was found to cause the reduction of MHC class I surface expression because the protein amounts of the MHC class I heavy chains were comparable in the whole cell lysate of DC from WT and double KO mice (de Verteuil *et al.*, 2010).

The immunoproteasome subunits play an important part in shaping the CD8 T cell repertoire. LMP2 and MECL-1 are found in complex with the thymus-specific $\beta 5t$ in the thymoproteasomes (Murata *et al.*, 2007), and both deficiency in LMP2 or MECL-1 may affect the generation of the T cell repertoire. The dominant response to influenza virus shifted from NP₃₆₆₋₃₇₄ and PA₂₂₄₋₂₃₃ in WT mice to NS₂₁₁₄₋₁₂₂ and PB1F₂₆₂₋₇₀ in LMP2 KO mice after i.p. infection. The adoptive transfer experiment with splenocytes followed by influenza infection showed that the immunodominance hierarchy of the WT or LMP2 KO donor cells was not influenced by the host genetic background, suggesting that the change of T cell repertoire in the LMP2 KO mice, not the change in peptide presentation, had caused the altered immunodominance hierarchy (Chen *et al.*, 2001b). The frequency of V β 10⁺ CD8 T cells was lower in MECL-1 KO mice and contributed to the reduced response to GP₂₇₆₋₂₈₆ after LCMV infection (Basler *et al.*, 2006). LMP7 is also expressed in the thymus (Murata *et al.*, 2007), and its absence influenced the selection of OT-1 CD8 T cells specific for an ovalbumin-derived H-2K^b-restricted peptide in that they were biased towards V β 8.1/8.2 usage instead of V β 5.1 in OT-1 LMP7 KO mice (Osterloh *et al.*, 2006).

The objective of this thesis is to study the generation of CD8 T cell response and specific memory during infections, the stability of memory T cells and their influences in subsequent heterologous infections. In the context of LCMV infection, I examine the effects of the absence of the immunoproteasome catalytic subunits on the magnitude and diversity of CD8 T cell response and the diversity of memory CD8 T cells. The stability of the pre-existing memory CD8 and CD4 T cells is re-examined using different infection sequences and a larger set of virus-specific peptides. I also examine the stability of Treg cells, which play an important role in autoimmunity, during LCMV infection. The influence of the history of infections is studied in LCMV-immune mice during persistent MCMV infection. Memory CD8 T cells are involved in the protecting LCMV-immune mice during VACV infection, and the non-reciprocity of immunoprotection between LCMV and VACV is investigated by comparing the memory phenotypes of memory CD8 T cells from LCMV and VACV infections and by increasing the potentially cross-reactive CD8 T cells in the VACV-immune mice.

Chapter II. Materials and Methods

Mice and viruses

C57BL/6 male mice between 5-6 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME). Foxp3GFP knock-in mice (from Dr. Vijay K. Kuchroo) (Bettelli *et al.*, 2006) and immunoproteasome LMP2/MECL1/LMP7 triple KO mice (from Dr. Kenneth Rock) (Kincaid *et al.*, 2011) were bred and maintained in a specific pathogen-free facility at the University of Massachusetts Medical School (UMMS)(Worcester, MA). Mice were used or received the first inoculum when they reached at least 6-7 weeks of age.

LCMV, Armstrong strain, was propagated in baby hamster kidney BHK21 cells (Welsh *et al.*, 1976; Welsh & Seedhom, 2008). MCMV, Smith strain, was passaged in BALB/c mice salivary glands (Selgrade *et al.*, 1982; Yang *et al.*, 1989). Supernatant from uninfected baby hamster kidney cultures (BHK) and salivary gland homogenate from naïve mice (nsg) were used as respective sham controls in some experiments. VACV, Western Reserve strain, was propagated in L929 cells (Yang *et al.*, 1989). Recombinant VACV expressing LCMV glycoprotein (VACV-GP) was kindly provided by Dr. J. Lindsay Whitton (Whitton *et al.*, 1988). Mice were infected with 5×10^4 pfu LCMV, 5×10^5 pfu MCMV, 10^6 pfu VACV or 5×10^6 pfu VACV-GP intraperitoneally, and were considered immune after 6 weeks post infection. Experiments were done in compliance with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research according to protocols approved by the Institutional Animal Care and Use Committee of UMMS.

Cell lines

Vero cells (African green monkey kidney epithelial cell line; ATCC) were maintained in minimum essential medium (MEM; Life Technologies) supplemented with 10% fetal calf serum (FCS; Sigma), 2mM Penicillin/Streptomycin (Pen/Strep; Gibco) and 2mM L-glutamine (L-glu; Gibco). Mouse embryonic fibroblasts from α -1-3-galactosyltransferase-deficient transgenic mice (GT-KO) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with 10% FCS, 2mM Pen/Strep and 2mM L-glu. DC2.4 (Shen *et al.*, 1997) were maintained in RPMI-1640 (Gibco) supplemented with 10% FCS, 2mM Pen/Strep, 2mM L-glu, non-essential amino acid (Gibco) and 10mM HEPES (Gibco).

DC immunization

Cultured immortalized dendritic cells DC2.4 were split 1:4 on the day before immunization. Trypsinized DC2.4 cells (at 10^7 cells/ml) were pulsed with 5 μ M filter-sterilized A11R₁₉₈₋₂₀₅ peptide in FCS in a 37°C water bath for 40 min with tapping every 10 min. Pulsed DC2.4 cells were washed 4 times with 50ml of Hank's Balanced Salt Solution (HBSS) and counted. Twenty-five million A11R₁₉₈₋₂₀₅-pulsed DC2.4 cells were administered subcutaneously on the upper right abdomen. HBSS was used as a control for the injection.

Plaque assay

Harvested organs were ground in 1ml of media (2ml for liver), spun at 2000 rpm for 20 min at 4°C, and stored at -80°C. Monolayers of Vero cells were set up overnight in 6-well plates by seeding 2×10^5 cells/well for LCMV or 3×10^5 cells/well for VACV. Organ homogenate (100 μ l) and its 10-fold dilutions were gently mixed onto and incubated with the monolayer in 1ml of media for 1.5 hrs at 37°C, 5% CO₂. Plates were then overlaid with 4ml of a 1:1 mixture of 1% SeaKem-ME agarose in distilled water and EMEM supplemented with 6% FCS, 5mM Pen/Strep, 5mM L-glu, and 1% Fungizone (Gibco). After 4 days for LCMV or 2 days for VACV, plates were stained with 0.02% neutral red in the agarose mixture above. Plaques were counted the following day.

Monolayers of GT-KO cells (2×10^5 cells/well) were used for MCMV plaque assays. After 6 days of incubation, the 4ml agarose overlay was carefully removed. The monolayer of GT-KO cells was washed with phosphate buffered saline (PBS), stained with 0.1% crystal violet with 1% formaldehyde in PBS, rinsed with PBS and air-dried.

Surface staining

Splenocytes in suspension were washed in staining buffer (1% FCS in PBS), blocked with anti-CD16/32 (clone 2.4G2; Fc block), and stained with combinations of anti-CD4 (clone RM4-5), anti-CD8 α (clone 53-6.7), anti-CD8 β (clone YTS156.7.7), anti-CD44 (clone IM7), anti-CD25 (clone PC61.5), anti-CD127 (clone A7R34), anti-CD122 (clone TM- β 1), anti-CD132 (clone TUGm2), anti-CD215 (clone JM7A4), anti-CD62L (clone MEL-14), anti-KLRG1 (clone 2F1), anti-CD27 (clone LG.3A10), anti-

CD43 (clone 1B11), anti-NK1.1 (clone PK136), anti-CD3 ϵ (clone 145-2C11), anti-V α 2 (clone B20.1), anti-Ly5.1 (clone A20), anti-Thy1.1 (clone HIS51), anti-Thy1.2 (clone 30-H12), live/dead fixable aqua (Invitrogen) for 20 min at 4°C. Stained cells were fixed with CytoFix (BD Biosciences) for 5 min at 4°C.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

To evaluate DNA fragmentation, cells were rested in media (10% FCS in RPMI-1640 with 2mM Pen/Strep and 2mM L-glu) supplemented with 10mM HEPES in a 48-well plate for 5 hrs at 37°C, 5%CO₂, Fc-blocked, surface stained, and Cytofixed for 15 min at 4°C. After permeabilization with 70% (v/v) ethanol for 3.5 days at -20°C, terminal deoxynucleotidyl transferase dUTP nick-end labeling was performed using Apo-BrdU-RedTM *In Situ* DNA Fragmentation Assay Kit (BioVision) according to manufacturer's instructions. Samples were analyzed within 3 hrs by LSRII (BD Biosciences) and FlowJo software (Tree Star).

Annexin V staining

Annexin V staining was done directly ex vivo. After Fc-block, surface staining and one wash with Annexin V binding buffer (eBioscience), cells were stained with Annexin V (eBioscience) at room temperature for 15 min. Samples were analyzed immediately by LSRII.

PhosFlow staining

Splenocytes in suspension were stimulated with IL-2 (5ng/ml; BD Biosciences) for 15 min at 37°C, 5%CO₂. After a quick wash in staining buffer, cells were immediately fixed with CytoFix for 15 min at 4°C, Fc-blocked and surface stained as described above. Cells were then permeabilized with PhosFlow Perm buffer III (BD Biosciences) for 30 min on ice, and stained with anti-STAT5 (clone 3H7) or anti-pSTAT5 (clone pY694) for 60 min at room temperature in the dark.

Ex vivo intracellular staining

Anti-apoptotic and pro-apoptotic molecules were examined without *in vitro* stimulation. Fc-blocked and surfaced-stained cells were fixed and permeabilized in Foxp3 Fix/Perm (eBioscience) for 1 hr at 4°C, washed with 1X Perm buffer and then stained with anti-Foxp3 (clone FJK-16s), anti-Bcl-2 (clone 3F11), anti-Bcl-xL (clone 54H6), anti-Mcl-1 (clone D35A5), anti-Bim (clone C34C5) or anti-Bak NT (Millipore #06-536) for 60 min at room temperature. Unconjugated antibodies were subsequently stained with Alexa Fluor 647 secondary goat anti-rabbit IgG (H+L) antibody (Life Technologies) for 30 min at room temperature in the dark.

Intracellular cytokine staining (ICS)

Splenocytes were stimulated on 96-well U-bottomed plates with 1µg/ml (1µM) synthetic peptide, 10 U/ml human recombinant IL-2 (BD Biosciences) and 1µl/ml of GolgiPlug (BD Biosciences) for 5 hrs at 37°C, 5% CO₂ as described (Brehm *et al.*, 2002). Anti-CD3ε (250ng/ml; clone 145-2C11), or 50ng/ml phorbol 12-myristate 13-acetate

(PMA) plus 500ng/ml ionomycin was used in place of peptides as positive controls. After Fc-block and surface staining as described above, cells were fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences) for 20 min at 4°C. Cells were then washed twice with 1X Perm/Wash buffer and stained with anti-IFN γ (clone XMG1.2), anti-TNF (clone MP6-XT22) and anti-IL-2 (clone JES6-5H4) for 25 min at 4°C. Washed samples were analyzed on an LSRII within 5 days. To assess the accumulation of lytic granules, samples were stimulated with GP₃₃₋₄₁ peptide for 4-6 days, and stained with anti-human Granzyme B (clone GB12) after fixation and permeabilization.

Proliferation assay with CFSE

Splenocytes were labeled with 2 μ M carboxyfluorescein succinimidyl ester (CFSE) in warm HBSS at 2×10^7 cells/ml for 15 min at 37°C with tapping every 5 min. Labeled splenocytes were washed in HBSS and counted. The Ly5.1 P14 CD8 T cell frequency of each mouse was determined by flow cytometry. Equal numbers of P14 memory CD8 T cells and supplementary naïve splenocytes were stimulated with GP₃₃₋₄₁ on a 96-well plate for 4-6 days. Proliferated populations were surface-stained and analyzed by LSRII.

In vivo brefeldin A (BFA) cytokine assay

Cytokine-producing cells were detected *in vivo* as described (Liu & Whitton, 2005; Priyadharshini *et al.*, 2010) with modifications. Lymphocytes from spleen and

lymph nodes of naïve Thy1.1 P14 TCR-transgenic mice specific for LCMV GP₃₃₋₄₁ were treated with 1 µl/ml GolgiPlug for 15-20 min at 37°C with tapping every 5 min. The BFA-treated lymphocytes (2×10^7) in 200 µl and 250 µg BFA in 200 µl warm HBSS were then injected intravenously into day 2 LCMV-infected wild-type and triple KO mice. Splenocytes were harvested after 4 h for ICS as described above.

Adoptive transfer experiment

WT Thy1.1+Thy1.2+ and triple KO Thy1.1+Thy1.2+ mice were injected with 40 µg anti-Thy1.2 (clone 30H12, BioXCell) per day on day -3, -2, -1 and +4 relative to infection to deplete endogenous T cells. LCMV-immune WT Thy1.1 splenocytes (3×10^7) were adoptively transferred intravenously on day -1, and mice were inoculated i.p. with 5×10^4 pfu LCMV on day 0. Splenocytes were harvested 5.5 days after infection for *in vitro* stimulation and ICS as described above.

DNA extraction and quantitative PCR (qPCR)

Organs collected in dry 1.5ml vials were snap-frozen in liquid nitrogen and stored at -80°C. A fraction of the snap-frozen organ was minced by glass beads in 10mM Tris/25mM EDTA (pH8.0) buffer, and incubated with proteinase K (Sigma) and 0.3% sodium dodecyl sulfate (Sigma) overnight in a 60°C water bath. After phenol extraction in phase lock gel (PLG) light tubes with equal volumes of the organ mixture and Tris-saturated phenol (pH 8.0), the aqueous phase was treated with 10-15u RNase (Promega) in a 37°C water bath for 1-1.5 hrs. DNA was extracted with phenol/chloroform in PLG

tubes, precipitated by adding 100% ethanol and 3M sodium acetate, and rinsed with 75% ethanol. Pelleted DNA was air-dried at room temperature before solvating in distilled water and storing at -20°C.

In a total volume of 25µl, the PCR mixture contained 125ng DNA, 400nM of forward primers, 400nM of reverse primers and QuantiFast SYBR Green PCR master mix (Qiagen). MCMV glycoprotein B (gB) forward primer sequence was 5'-AGGGCTTGGAGAGGACCTACA-3' and reverse primer sequence was 5'-GCCCGTCGGCAGTCTAGTC-3' (Vliegen *et al.*, 2003). Mouse β-actin forward primer sequence was 5'-CGAGGCCAGAGCAAGAGAG-3' and reverse primer sequence was 5'-CGGTTGGCCTTAGGGTTCAG-3' (Mishra *et al.*, 2010). The PCR was performed on CFX96 (BioRad) with an initial incubation at 95°C for 10 min to activate the Taq enzyme followed by 35 amplification cycles of denaturation at 95°C for 10s, annealing and extension at 60°C for 30s. A melting curve was generated from 65°C to 95°C with an increment of 0.5°C every 5s. Serial dilutions of plasmid containing the gB sequence ($10^9 - 10^2$ copies) were included to generate a standard curve. The β-actin standards contained 375-0.17ng genomic DNA. MCMV copy number was calculated by the CFX System (Bio-Rad) in duplicates and normalized with the amount of genomic DNA in µg.

Statistical analysis

Student's t test was calculated using Excel or Prism. Data were presented with mean and standard error of the mean (SEM).

Chapter III. Immunoproteasomes influence immune responses

(The study in this chapter was accomplished in collaboration with Eleanor Kincaid, and part of the work has already been published in *Nature Immunology* 13(2):129-135.)

The magnitude and diversity of CD8 T cell response to an infection can be affected by the peptides generated in the immunoproteasomes and presented on MHC class I molecules by the antigen-presenting cells. Professional antigen-presenting cells express both constitutive proteasomes and immunoproteasomes (Nil *et al.*, 2004). The 20S core of the proteasomes consists of 14 α -type subunits on the two outer rings and 14 β -type subunits on the inner rings. The catalytically active β -type subunits δ (β 1), MB1 (β 5) and MC14 (β 2) are constitutively expressed but are replaceable by the inducible homologues LMP2 (β 1i), LMP7 (β 5i), and MECL-1 (β 2i) upon IFN γ stimulation on non-hematopoietic cells (Aki *et al.*, 1994; Coux *et al.*, 1996; Nandi *et al.*, 1996). The inducible catalytic subunits incorporated into the newly assembled immunoproteasomes can modulate the enzymatic activities and substrate sequence preferences (Boes *et al.*, 1994; Gaczynska *et al.*, 1993). In some cells, LMP2 and MECL-1 are incorporated cooperatively into the immunoproteasomes, and the maturation of LMP2 and MECL-1 progresses more efficiently in the presence of the presequence of LMP7 (Griffin *et al.*, 1998; Groettrup *et al.*, 1997; Pang *et al.*, 2006). Single allele disruption knockout mice have been generated to study the function of the inducible immunoproteasome subunits separately (Basler *et al.*, 2006; Fehling *et al.*, 1994; Van Kaer *et al.*, 1994). The LMP7/MEC-1 and LMP2/MEC-1 double KO mice were subsequently made by genetic crosses (Hensley *et al.*, 2010; Pang *et al.*, 2006). Because of the close proximity of the

genes encoding LMP2 and LMP7 (*Psmb9* and *Psmb8* respectively) in the MHC class II region, a LMP2/LMP7 double KO is impossible to achieve by normal crosses of the single KO mice. Therefore, Eleanor Kincaid and colleagues generated the LMP2/LMP7 double KO mouse by sequential target deletion of exons 1-5 of *Psmb8* and exon 1 of *Psmb9*. The backcrossed LMP2/LMP7 double KO mouse with the C57BL/6J background was then bred with MECL-1 KO mice to generate the LMP2/LMP7/MECL-1 triple KO mice.

The phenotype of the triple KO mice is a combination of the characteristics of the three single KO strains. Similar to LMP2/MECL-1 and LMP7/MECL-1 double KO mice (Hensley *et al.*, 2010), the mature CD8 T cell count in the thymus and spleen of the triple KO mice is only about half of that in WT mice, but the B cell number is normal. This is different from LMP2 single KO mice, whose B cells as well as CD4 and CD8 T cells are reduced (Hensley *et al.*, 2010; Nussbaum *et al.*, 2005). The LMP7 KO reduced the presence of MHC class I molecules on cell surface (Fehling *et al.*, 1994), but the LMP2 KO or MECL-1 KO did not (Basler *et al.*, 2006; Van Kaer *et al.*, 1994). The triple KO mice are more similar to the LMP7 KO mice in this aspect and express only about 50% as many MHC class I molecules H-2K^b and H-2D^b as WT hematopoietic cells do. Expression of *Tap1*, which is located between *Psmb9* and *Psmb8*, is also reduced by 50% in the triple KO mice, but *Tap1* expression does not affect MHC class I surface expression (Kincaid *et al.*, 2011). Peptides eluted from the triple KO and WT splenocytes are substantially different, consistent with the previous observation with double-deficient and WT immunoproteasomes (de Verteuil *et al.*, 2010; Toes *et al.*,

2001). Adoptively transferred WT splenocytes are rejected by the triple KO hosts in several days, but the triple KO splenocytes survive in the WT hosts, since the presence of both constitutive proteasomes and immunoproteasomes in the WT mice would allow WT mice to tolerate peptides generated by both complexes.

Previous studies of LCMV infection in single KO mice revealed normal CD8 T cell responses to the major epitopes in the LMP7 KO mice, but the responses to GP₂₇₆₋₂₈₆ and NP₂₀₅₋₂₁₂ were reduced in LMP2 KO and MECL-1 KO mice (Basler *et al.*, 2006; Nussbaum *et al.*, 2005). In this study, I evaluated the CD8 T cell response to LCMV and the generation of LCMV-specific memory CD8 T cells in the triple KO mice. I also examined the activation of WT CD8 T cells in the triple KO hosts during LCMV infection.

Results

Reduced LCMV-specific CD8 T cell response in triple KO mice

To examine the CD8 T cell response to LCMV, age-matched LMP2/LMP7/MECL-1 triple KO, MECL-1 KO and WT mice were infected with 5×10^4 pfu LCMV Armstrong, and splenocytes were analyzed at 9 days post infection by ICS. As previously reported (Basler *et al.*, 2006), the CD8 T cell response to GP₂₇₆₋₂₈₆ and NP₂₀₅₋₂₁₂ was significantly reduced in MECL-1 KO mice (Fig. 3.1). In the triple KO mice, not only the GP₂₇₆₋₂₈₆- and NP₂₀₅₋₂₁₂-specific CD8 T cell responses but also the GP₃₃₋₄₁-, GP₃₄₋₄₁-, and GP₁₁₈₋₁₂₅-specific CD8 T cell responses were significantly reduced both in frequencies (Fig. 3.1a) and in numbers (Fig. 3.1b). On the other hand, NP₃₉₆₋₄₀₄-specific CD8 T cell responses in the triple KO mice were comparable to the WT mice. As a result, the immunodominance hierarchy was shifted in the triple KO mice, and more than 50% of the CD8 T cell response was specific for NP₃₉₆₋₄₀₄ (Fig. 3.1c).

Although the overall magnitude of the CD8 T cell response to LCMV was much reduced, the CD4 T cell response was not affected in the triple KO or the MECL-1 KO mice (Fig. 3.2a), and the LCMV viral titer in the spleen was not significantly different between the triple KO mice and the WT controls at day 5 post infection (Fig. 3.2b), indicating comparable antigen load between the two genotypes, at least under the conditions of this relatively low dose inoculum.

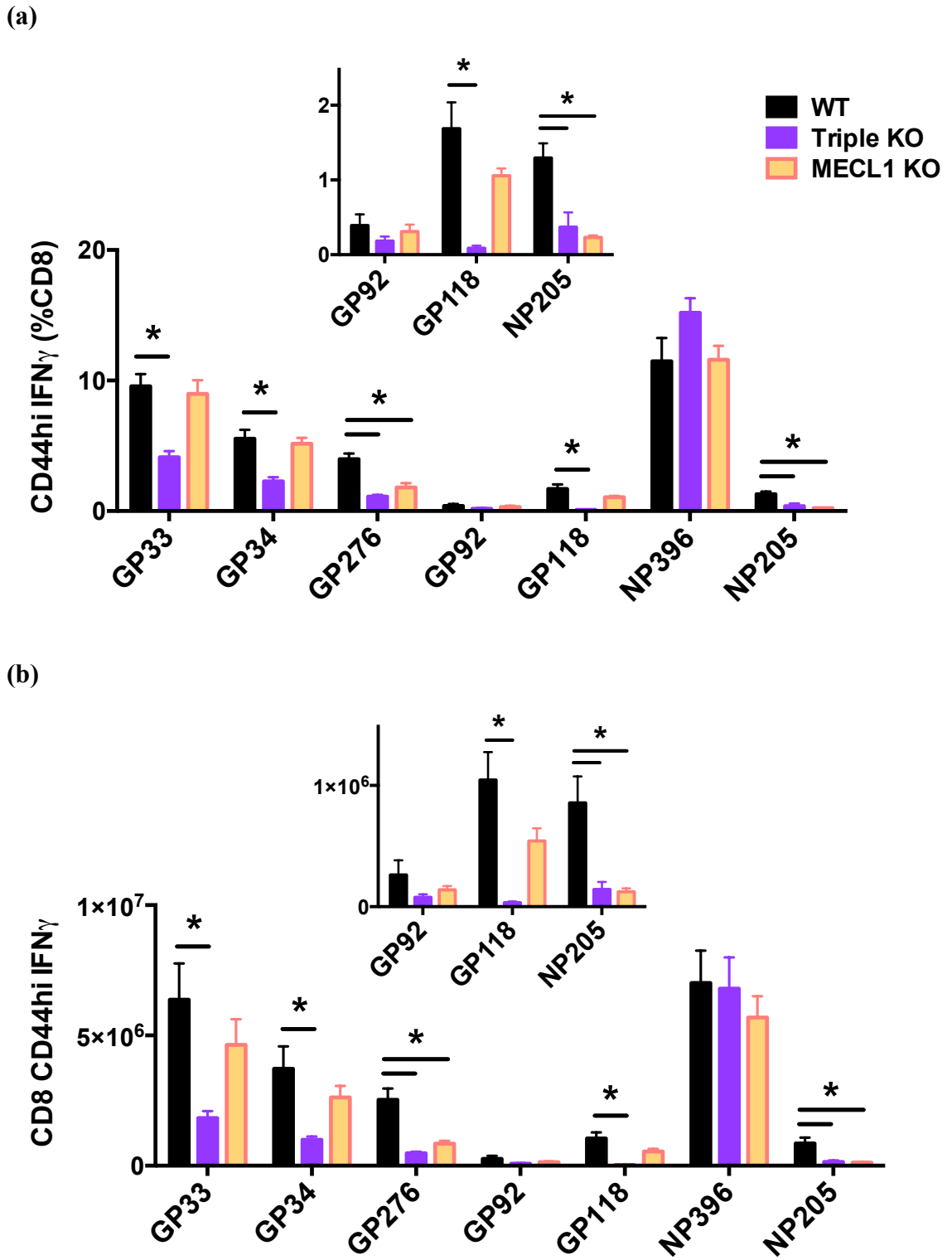


Figure 3.1.

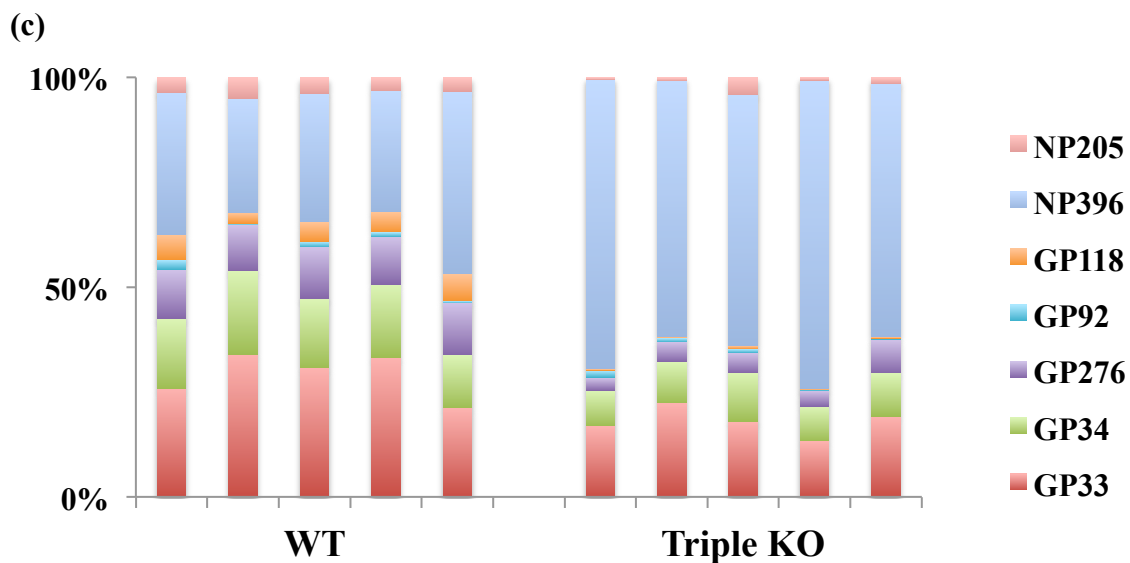


Figure 3.1. The absence of the immunoproteasome reduces the CD8 T cell response to LCMV and alters the immunodominance hierarchy. WT and triple KO mice were infected i.p. with 5×10^4 pfu LCMV. The frequency (a) and number (b) of LCMV-specific CD8 T cells were determined at 9 days post infection by ICS. (c) The epitope-specific CD8 T cell response at day 9 post infection was displayed in a stack plot to show the altered immunodominance hierarchy in the triple KO mice. Data are representative of at least two separate experiments. (*) denotes statistical significance ($p < 0.05$) between WT and KO groups ($n=5$).

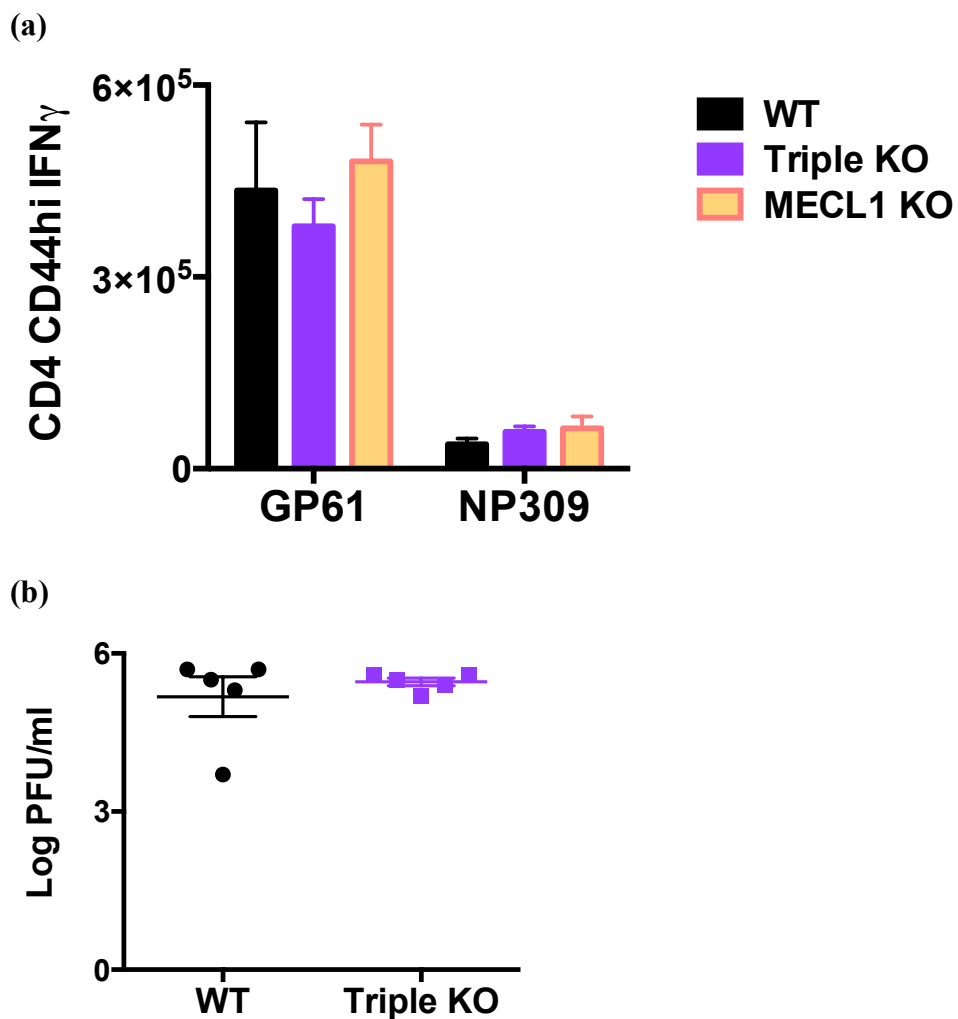


Figure 3.2. The absence of immunoproteasome subunits does not affect CD4 T cell response or LCMV viral titer. WT, triple KO and MECL-1 KO mice were infected i.p. with 5×10^4 pfu LCMV. (a) LCMV GP₆₁₋₈₀- and NP₃₀₉₋₃₂₈-specific CD4 T cell responses were determined at 9 days post infection by ICS. (b) Whole spleens from WT and triple KO mice were collected for viral titer at 5 days post infection. Data are representative of at least two separate experiments (n=5).

Impaired antigen presentation in triple KO mice

The reduced immune response may have reflected defects in antigen presentation, in T cell proliferation or in the T cell repertoire. To evaluate if there was a problem with antigen presentation in the triple KO mice, I examined T cell activation in the WT and the triple KO mice by *in vivo* cytokine assay. BFA-treated lymphocytes from the spleen and the lymph nodes of WT P14 mice were adoptively transferred into day 2 LCMV-infected WT and triple KO hosts and examined 4 hrs later by ICS. In this study, a significantly lower percentage of the donor P14 CD8 T cells were activated in the infected triple KO hosts than in the infected WT hosts, as measured by the down-regulation of CD62L and the production of TNF (Fig. 3.3a) and IFN γ (Fig. 3.3b), suggesting that the GP₃₃₋₄₁ peptide was not presented as efficiently in the triple KO hosts as in the WT hosts. Rejection of WT cells should not be a problem in this short assay.

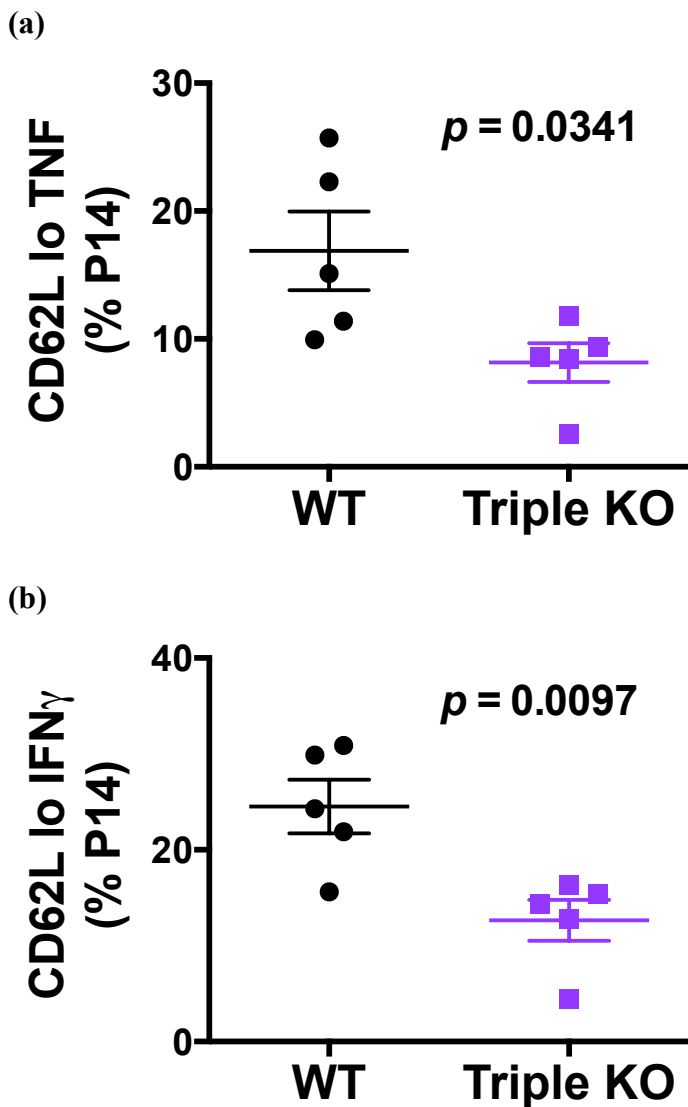


Figure 3.3. P14 CD8 T cells are less activated in the day 2 LCMV-infected immunoproteasome triple KO hosts. BFA-treated P14 lymphocytes were adoptively transferred into day 2 LCMV-infected WT and triple KO hosts along with a separate injection of 250 μ g BFA in 200 μ l warm HBSS. Splenocytes were harvested after 4 hrs and analyzed by ICS. The frequencies of activated CD62L lo TNF⁺ (a) and CD62L lo IFN γ ⁺ (b) within the Thy1.1 P14 CD8 T cell populations were compared. Data (with mean and SEM) are representative of two separate experiments.

A proliferative response can be another measure for activation. Initial attempts to compare the proliferation of CD8 T cells by adoptive transfer experiments were impaired by low recovery of donor cells. LCMV might have served as an adjuvant and accelerated the allospecific rejection of the transferred cells. To get around the host-versus-graft allogeneic barrier, CD8 T cells were depleted from WT and triple KO hosts with three consecutive injections of anti-Thy1.2 antibody before adoptive transfer of WT Thy1.1+ splenocytes. On day 5.5 after i.p. infection with 5×10^4 pfu LCMV, splenocytes were analyzed with ICS. A negligible number of host T cells was found in the spleen after depletion. Comparing to the WT hosts, the specific donor CD8 T cell responses to GP₃₃₋₄₁, GP₃₄₋₄₁ and GP₁₁₈₋₁₂₅ were significantly reduced in the triple KO hosts, while the specific responses to NP₃₉₆₋₄₀₄, GP₉₂₋₁₀₁, and NP₂₀₅₋₂₁₂ were not significantly different (Fig. 3.4a). In contrast, the GP₂₇₆₋₂₈₆-specific CD8 T cell response was significantly elevated in the triple KO hosts. On the other hand, the donor CD4 T cell responses to GP₆₁₋₈₀ and NP₃₀₉₋₃₂₈ were not significantly affected by the deficiency of the immunoproteasome subunits, although the response to NP₃₀₉₋₃₂₈ seemed slightly elevated in the triple KO hosts (Fig. 3.4b). Therefore, the absence of the immunoproteasome subunits can significantly change the presentation of a number of LCMV epitopes and affect the activation of CD8 T cells.

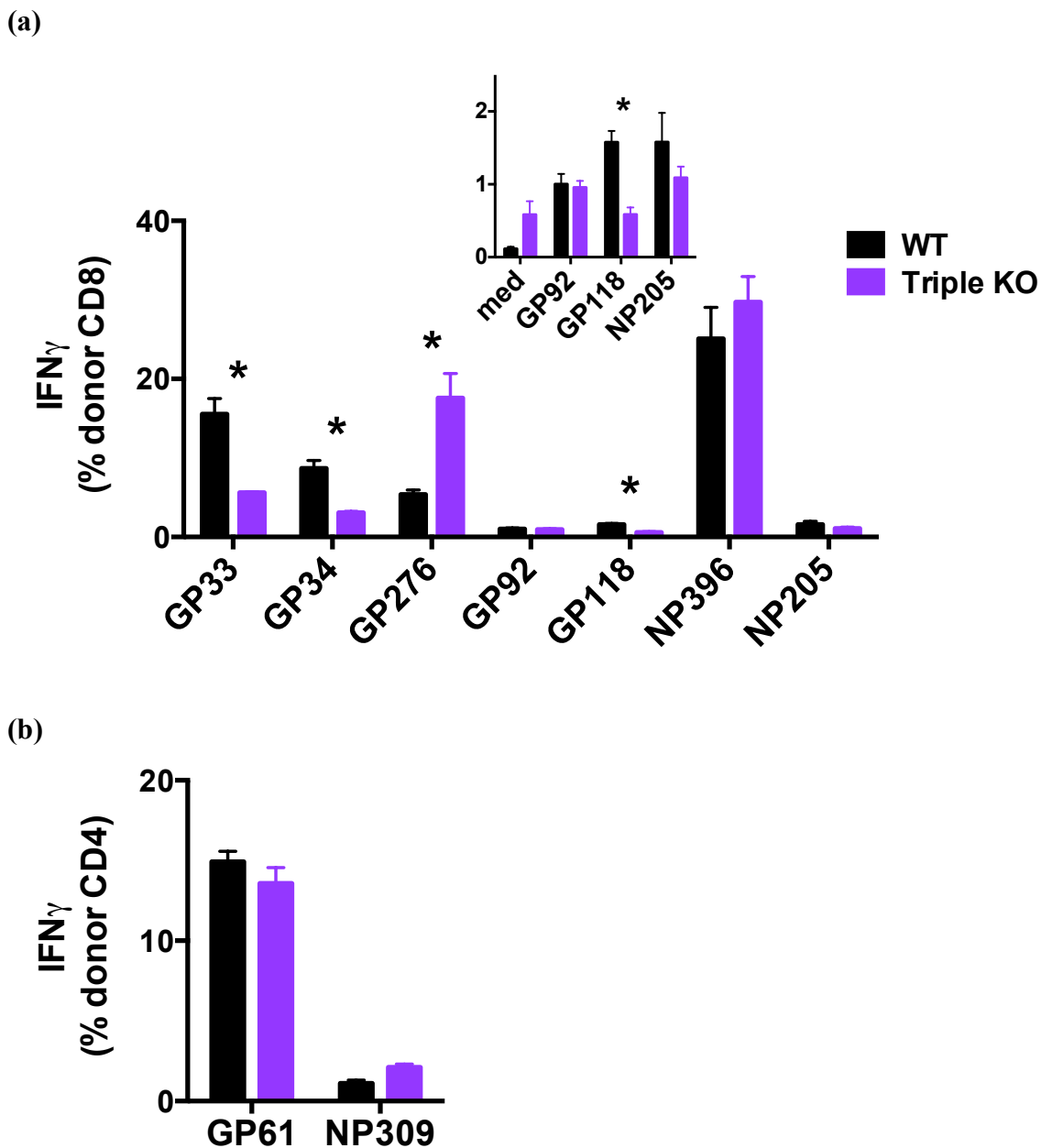


Figure 3.4. WT CD8 T cells differentially proliferate less in response to LCMV infection in the immunoproteasome KO hosts. CD8 T cells were depleted from WT and triple KO hosts with three consecutive injection of anti-Thy1.2 antibody beginning three days before infection. WT Thy1.1⁺ splenocytes were adoptively transferred one day before i.p. infection with 5×10^4 pfu LCMV. Donor CD8 (a) and CD4 (b) T cells in the spleens were analyzed by ICS on day 5.5 post infection. (*) denotes statistical significance ($p < 0.05$) between WT and triple KO host groups in the combined data from two experiments (n=6).

Reduced LCMV-specific memory CD8 T cells in triple KO mice

To examine if the absence of the immunoproteasome subunits would affect the generation of memory CD8 T cells, WT and triple KO mice were infected with 5×10^4 pfu LCMV, and the splenocytes were harvested at 10 weeks post infection for analysis by ICS after *in vitro* peptide stimulation. I found that the triple KO mice generated a significantly reduced number of GP₃₃₋₄₁- and GP₂₇₆₋₂₈₆-specific memory CD8 T cells, and a minimal number of GP₉₂₋₁₀₁-, GP₁₁₈₋₁₂₅- and NP₂₀₅₋₂₁₂-specific memory CD8 T cells at week 10 post LCMV infection, while the NP₃₉₆₋₄₀₄-specific memory CD8 T cells were not affected (Fig. 3.5a). Surprisingly, the GP₆₁₋₈₀-specific memory CD4 T cells were more abundant in the triple KO mice, but the NP₃₀₉₋₃₂₈-specific memory CD4 T cells were not different from the WT mice (Fig. 3.5b). A similar pattern was observed at 29 weeks post LCMV infection.

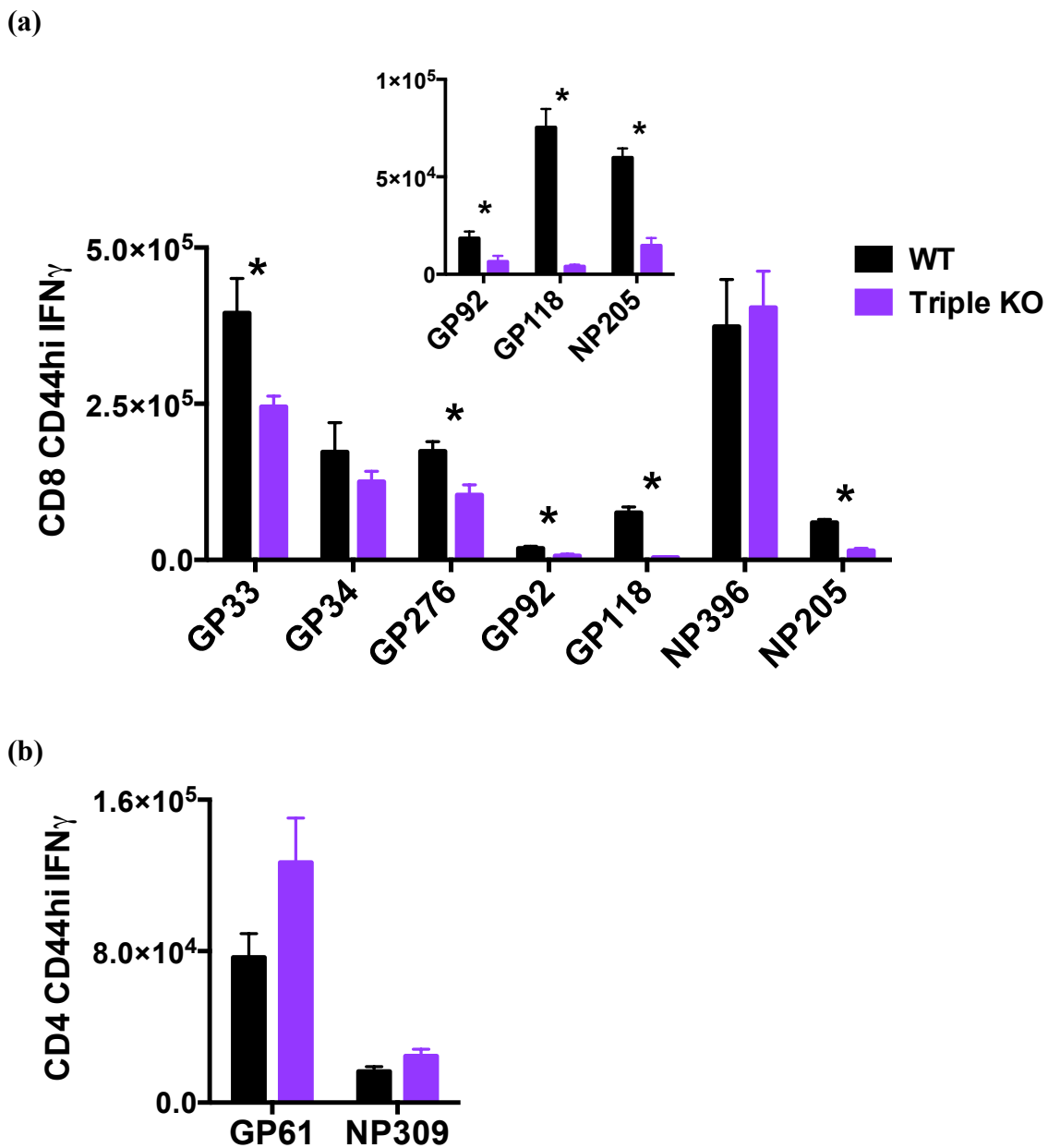


Figure 3.5. The generation of LCMV epitope-specific memory T cells is differentially affected by the absence of the immunoproteasome subunits. WT and triple KO mice were infected i.p. with 5×10^4 pfu LCMV. Memory CD8 (a) and CD4 (b) T cells were analyzed by ICS at 10 weeks post infection. (*) denotes statistical significance ($p < 0.05$) between WT and triple KO groups ($n=4$).

Discussion

In this study, the overall magnitude of the CD8 T cell response to LCMV was considerably reduced in the triple KO mice as compared to the WT controls. Previous studies with the immunoproteasome subunit single KO mice showed only modest changes in the CD8 T cell response to LCMV infection (Basler *et al.*, 2006; Nussbaum *et al.*, 2005). The defects in the triple KO mice were much more profound than those in the LMP2, LMP7 and MECL-1 single KO mice combined. In addition to the reduced response to GP₂₇₆₋₂₈₆⁻ and NP₂₀₅₋₂₁₂ previously reported in the LMP2 and the MECL-1 single KO mice (Basler *et al.*, 2006; Nussbaum *et al.*, 2005), the CD8 T cell response to GP₃₃₋₄₁⁻, GP₃₄₋₄₁⁻, GP₁₁₈₋₁₂₅, and GP₉₂₋₁₀₁ were all reduced in the triple KO mice. The synergistic nature of the KO phenotypes suggested that the immunoproteasome subunits were functionally interrelated. As a result, the immunodominance hierarchy was shifted, with more than 50% of the total CD8 T cell response being NP₃₉₆₋₄₀₄-specific. Immunodominance hierarchy is influenced by multiple factors, including protein expression kinetics (Probst *et al.*, 2003), peptide abundance (Gallimore *et al.*, 1998), binding affinity between peptide and MHC molecules, and T cell precursor frequency (Kotturi *et al.*, 2008). The absence of the immunoproteasome subunits probably influences the immunodominance hierarchy through changing the peptide repertoire and abundance in the antigen-presenting cells, and through altering the T cell repertoire during T cell development.

The acute response and the generation of memory CD8 T cells specific for most of the major LCMV epitopes were impaired in the triple KO mice, and the GP₁₁₈₋₁₂₅-

specific memory CD8 T cells were completely missing. The WT donor CD8 T cell response specific for GP₃₃₋₄₁, GP₃₄₋₄₁, GP₁₁₈₋₁₂₅ and NP₂₀₅₋₂₁₂ in the triple KO hosts was weaker than that in the WT hosts, suggesting that the impaired peptide presentation in the absence of the immunoproteasome subunits was causing the reduced response to LCMV infection. Presentation of GP₁₁₈₋₁₂₅ is probably completely dependent on the immunoproteasome subunits because the WT CD8 T cell response to GP₁₁₈₋₁₂₅ in the triple KO hosts was so close to the background reading (Fig. 3.4a). That would also explain the nearly non-existent GP₁₁₈₋₁₂₅-specific response on day 9 post LCMV infection and the missing GP₁₁₈₋₁₂₅-specific memory CD8 T cells at 10 weeks post infection.

The presentation of the NP₂₀₅₋₂₁₂ epitope reveals the complexity of the interrelated functionality of the immunoproteasome subunits. Previous studies in the single KO mice suggested that both LMP2 and MECL-1 are required for the presentation of NP₂₀₅₋₂₁₂ but that LMP7 is dispensable (Basler *et al.*, 2006; Nussbaum *et al.*, 2005). A recent study using a LMP7-specific inhibitor, ONX 0914, showed that blocking the chymotrypsin-like activity of LMP7 can rescue the presentation of NP₂₀₅₋₂₁₂ in the LMP2/MECL-1 double KO mice while this inhibitor has no effect in the WT mice (Basler *et al.*, 2011).

However, NP₂₀₅₋₂₁₂ was suboptimally presented in the triple KO mice in this study.

These data suggest that in order to optimally generate the NP₂₀₅₋₂₁₂ peptide, LMP2 and MECL-1 have to be present in the same proteasome, but the pairing of the constitutive homologs δ (in place of LMP2) and MC14 (in place of MECL-1) is also allowed. The proteasome with LMP2 and MECL-1 can generate NP₂₀₅₋₂₁₂ in the presence of LMP7, the chemically inactivated LMP7, or the constitutive homolog MB1, as the use of ONX 0914

in WT mice and the LMP7 deficiency have no effect on the generation of NP₂₀₅₋₂₁₂.

However, the proteasomes containing the constitutive subunits δ and MC14 can produce NP₂₀₅₋₂₁₂ only when LMP7 (and not MB1) is present but chemically inactivated.

The NP₃₉₆₋₄₀₄-specific CD8 T cell response and memory generation were not affected by the absence of immunoproteasome subunits. One possibility is that the NP₃₉₆₋₄₀₄ peptide can be generated by the constitutive proteasomes alone. This may be part of the reasons why the adoptively transferred NP₃₉₆₋₄₀₄-specific CD8 T cell line can reduce LCMV titer in mice most efficiently (Gallimore *et al.*, 1998), because any infected cells can become the targets of the cytotoxic T cells without any help of the cytokines.

Alternatively, the apparent immunoproteasome independence may reflect an overwhelmed presentation system, where the great abundance of the nucleoprotein early during infection (Bruns *et al.*, 1990; Probst *et al.*, 2003) and the high MHC binding affinity of the NP₃₉₆₋₄₀₄ peptide (Kotturi *et al.*, 2008) were masking any effect of the absence of the immunoproteasome subunits.

Both the reduced peptide generation and the altered T cell repertoire may have contributed to the reduced GP₂₇₆₋₂₈₆-specific CD8 T cell response in the triple KO mice. The adoptively transferred WT splenocytes mounted a greater response to GP₂₇₆₋₂₈₆ in the triple KO hosts than in the WT hosts, suggesting a better presentation of GP₂₇₆₋₂₈₆ in the triple KO hosts. This is consistent with the previous report that LMP2 in the immunoproteasome could destroy the GP₂₇₆₋₂₈₆ peptide *in vitro* (Basler *et al.*, 2004). However, the GP₂₇₆₋₂₈₆-specific CD8 T cell response was reduced in the LMP2 KO mice (Nussbaum *et al.*, 2005) and in the triple KO mice in this study. A lower percentage of

V β 10b+ CD8 T cells in the triple KO mice after LCMV infection was previously reported (Basler *et al.*, 2006). A similar observation was made in this study; however, no significant difference in the V β usage was found among the GP₂₇₆₋₂₈₆-tetramer-positive CD8 T cells at 8 days post LCMV infection (data not shown). V β usage was not different among GP₃₃₋₄₁-, GP₃₄₋₄₁-, and NP₃₉₆₋₄₀₄-tetramer-positive CD8 T cells, either. Nonetheless, both the nature of the peptide presentation and the T cell repertoire probably have certain influences in the reduction of GP₂₇₆₋₂₈₆-specific CD8 T cell response in the triple KO mice. On the other hand, the altered T cell repertoire likely played a greater role in the reduction of GP₉₂₋₁₀₁-specific CD8 T cell response in the triple KO mice, as the WT CD8 T cell response to GP₉₂₋₁₀₁ was comparable in both WT and the triple KO hosts, suggesting no apparent defects in the presentation of GP₉₂₋₁₀₁ peptide in the triple KO hosts.

The immunoproteasomes are important in the generation of peptides for MHC class I presentation. Both constitutive proteasomes and immunoproteasomes are expressed in the medullary thymic epithelial cells (Nil *et al.*, 2004) and can therefore influence the negative selection of T cells during development. Antigen-presenting cells such as DC and macrophages constantly express the immunoproteasomes as well as the constitutive proteasomes (Nil *et al.*, 2004). Therefore, the immunoproteasomes can influence the generation of the T cell repertoire, self-tolerance and immune response. Previous reports using the single KO mice substantially underestimated the contributions of the immunoproteasomes during infections (Basler *et al.*, 2006; Nussbaum *et al.*, 2005). As demonstrated in this study, the immunoproteasomes not only increase the magnitude

of CD8 T cell response during infection but also diversify the specificity such as by enabling the generation of GP₁₁₈₋₁₂₅-specific response. Therefore, the immunoproteasomes are crucial in viral control and immunity.

Chapter IV. Attrition of memory CD8 and CD4 T cells during heterologous infections

(Part of the data has been published in *Immunol Rev* 235 (1): 244-266)

Memory CD8 T cells generated upon encounter with pathogens are generally stable but memory CD4 T cells may decline over time (Homann *et al.*, 2001; Selin *et al.*, 1996; 1999). The stability of memory CD8 T cells, however, can be affected by heterologous infections with unrelated pathogens. The reduction of memory CD8 T cells has been previously demonstrated in various viral infection sequences by limiting dilution assay, intracellular cytokine staining, MHC-dimer or tetramer staining and *in vivo* cytotoxicity assays:

LCMV + PV	(Brehm <i>et al.</i> , 2002; Selin <i>et al.</i> , 1996; 1999; Varga <i>et al.</i> , 2001)
LCMV + PV + VACV	(Selin <i>et al.</i> , 1996; 1999; Varga <i>et al.</i> , 2001)
LCMV + PV + MCMV	(Selin <i>et al.</i> , 1996)
LCMV + PV + VACV + MCMV	(Selin <i>et al.</i> , 1996; 1999; Varga <i>et al.</i> , 2001)
LCMV + MCMV	(Selin <i>et al.</i> , 1996; 1999)
PV + LCMV	(Kim & Welsh, 2004)
Vesicular stomatitis virus + LCMV	(Kim & Welsh, 2004)
VACV + LCMV	(Kim & Welsh, 2004)
VACV + MCMV	(Selin <i>et al.</i> , 1996)
Influenza + γ -herpesvirus 68	(Liu <i>et al.</i> , 2003)

Bacterial infections can also cause a reduction of memory CD8 T cells, as it has been shown in BCG- or *Salmonella typhimurium*-challenged LM-immune mice (Dudani *et al.*, 2008; Smith *et al.*, 2002). A similar phenomenon has been reported in humans as well. Patients acutely infected with hepatitis B virus were found to have reduced number of cytomegalovirus-specific CD8 T cells than the healthy (hepatitis B negative) cohort (Zhang *et al.*, 2008).

IFNs are associated with attrition of memory CD8 T cells. Previous work with viruses has shown that the reduction of pre-existing memory T cells is associated with type I IFN, which is induced early during some infections and causes apoptosis in CD44^{hi} CD8 T cells (McNally *et al.*, 2001). After infection with a good type I IFN-inducing pathogen, like LCMV, pre-existing memory CD8 T cells will be reduced early after infection and remain stable at a lower number after a minor recovery (Bahl *et al.*, 2006; Kim & Welsh, 2004). On the other hand, IFN γ is found associated with memory CD8 T cell attrition caused by bacterial infections (Dudani *et al.*, 2008).

A subsequent publication suggested that the CD8 compartment is expandable and challenged the phenomenon of attrition by arguing that the erosion of pre-existing memory CD8 T cells may be substantial in percentage but much less in actual numbers (Vezys *et al.*, 2009). In response to that, previous work was recalculated to show the reduction in number of LCMV-specific memory CD8 T cells after subsequent viral challenges (Welsh & Selin, 2009). Additionally, I performed a series of new experiments to re-examine this phenomenon of memory reduction using other virus combinations and

to examine the memory CD8 T cells with a larger set of viral epitope specificities. In some of these experiments, where peptides were available, I also analyzed the stability of memory CD4 T cells.

Results

Reduction of LCMV-specific memory T cells by MCMV infection

Like LCMV, MCMV induces an IFN $\alpha\beta$ response early during infection (Schneider *et al.*, 2008). To examine the effect of MCMV infection on LCMV-specific memory CD8 and CD4 T cells, C57BL/6 mice were inoculated with 5×10^4 pfu LCMV and rested for 6 weeks to allow T cells to return to homeostasis. LCMV-immune mice were subsequently infected with 5×10^5 pfu MCMV or given the salivary gland homogenate from naïve animals as a sham control. Compared to LCMV-immune mice without MCMV infection, splenic memory CD8 T cells specific for LCMV dominant epitopes GP₃₃₋₄₁, NP₃₉₆₋₄₀₄ and subdominant epitopes GP₂₇₆₋₂₈₆, GP₁₁₈₋₁₂₅ and NP₂₀₅₋₂₁₂ at 9 weeks post MCMV infection were reduced in number and in frequency (Fig. 4.1a and 4.1b). A similar reduction was also observed in the bone marrow of the same animals (Fig. 4.1c). Due to the low and variable number of cells collected and rarity of T cells in the bone marrow, only the reduction of GP₃₃₋₄₁-specific memory CD8 T cell numbers reached statistical significance, although the decrease in percentages of memory CD8 T cells of other specificities were statistically significant in the bone marrow (Fig. 4.1d). MCMV infection also significantly reduced the memory CD4 T cell population in the spleen, causing about a 50% reduction in number and in frequency of GP₆₁₋₈₀-specific memory CD4 T cells (Fig. 4.2a and 4.2b). NP₃₀₉₋₃₂₈-specific CD4 T cells were also significantly reduced in percentage in the spleen.

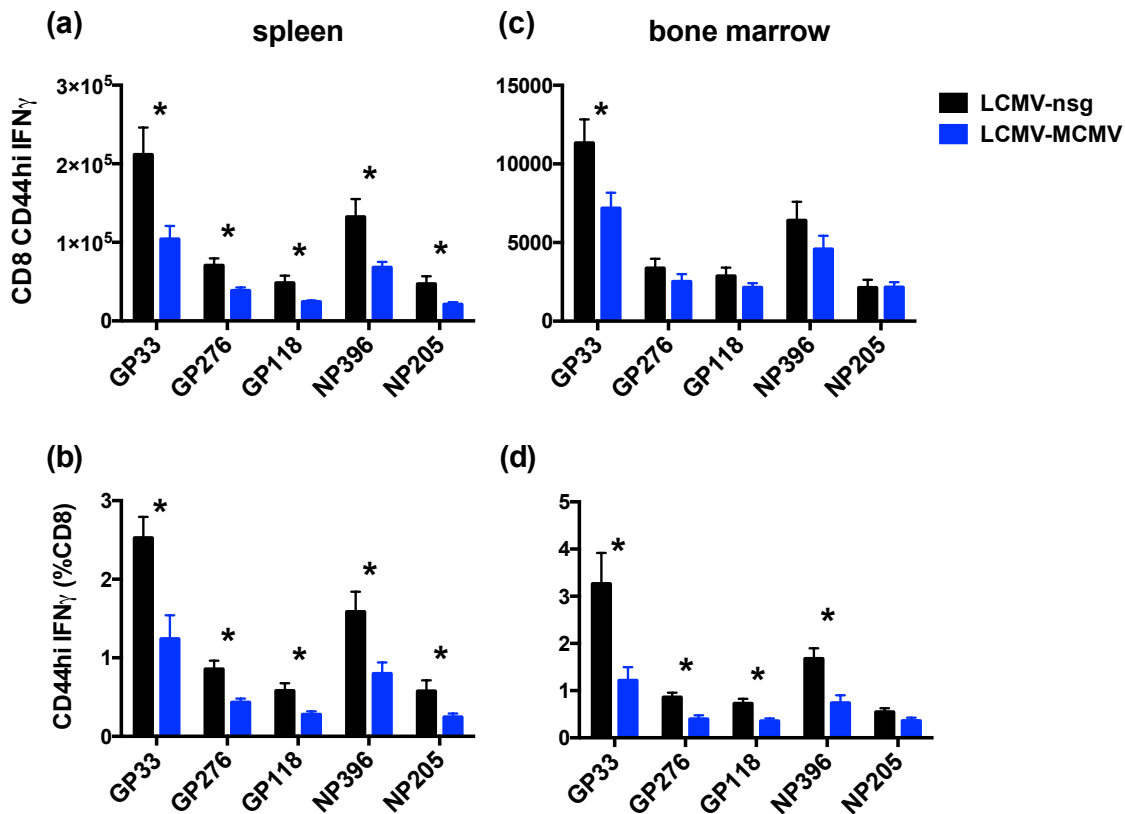


Figure 4.1. MCMV infection significantly reduces the number and frequency of LCMV-specific memory CD8 T cells. LCMV-immune mice were infected i.p. with 5×10^5 pfu MCMV or given salivary gland homogenate from naïve mice as sham control. Memory CD8 T cells from the spleen (a and b) and bone marrow (c and d) were examined at 9 weeks post MCMV infection by ICS. (*) denotes statistical significance ($p < 0.05$) between MCMV-infected and nsg control groups (n=5).

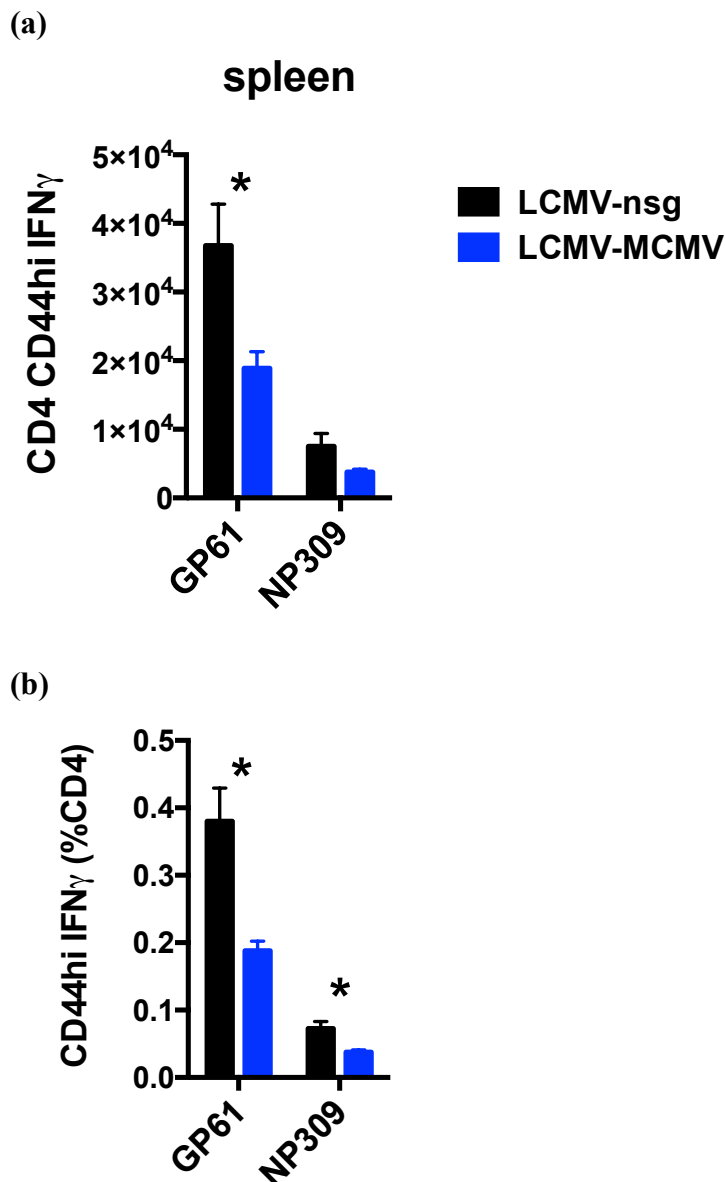


Figure 4.2. MCMV infection significantly reduces the number and frequency of LCMV-specific memory CD4 T cells. LCMV-immune mice were infected i.p. with 5×10^5 pfu MCMV or given salivary gland homogenate from naïve mice as sham control. Memory CD4 T cells from the spleen were examined at 9 weeks post MCMV infection by ICS. (*) denotes statistical significance ($p < 0.05$) between MCMV-infected and nsg control groups ($n=5$).

Reduction of VACV-specific memory T cells by LCMV and MCMV infections

The attrition of VACV-specific memory T cells was previously examined using VACV-infected stimulator peritoneal exudate cells in ICS assays (Kim & Welsh, 2004). In this study, VACV-derived peptide stimulation was used in ICS to identify the VACV epitope-specific memory CD8 T cell populations. Mice inoculated with 10^6 pfu VACV and rested for 6 weeks were infected i.p. with 5×10^4 pfu LCMV or given the supernatant from uninfected BHK cell cultures as a sham control. At 9 weeks post LCMV infection, the numbers and frequencies of pre-existing VACV B8R₂₀₋₂₇- and E7R₁₃₀₋₁₃₇-specific memory CD8 T cells were significantly reduced in the spleen (Fig. 4.3a and 4.3b). A similar reduction of memory CD8 T cells was also observed in the peritoneum of the same mice (Fig. 4.3c and 4.3d).

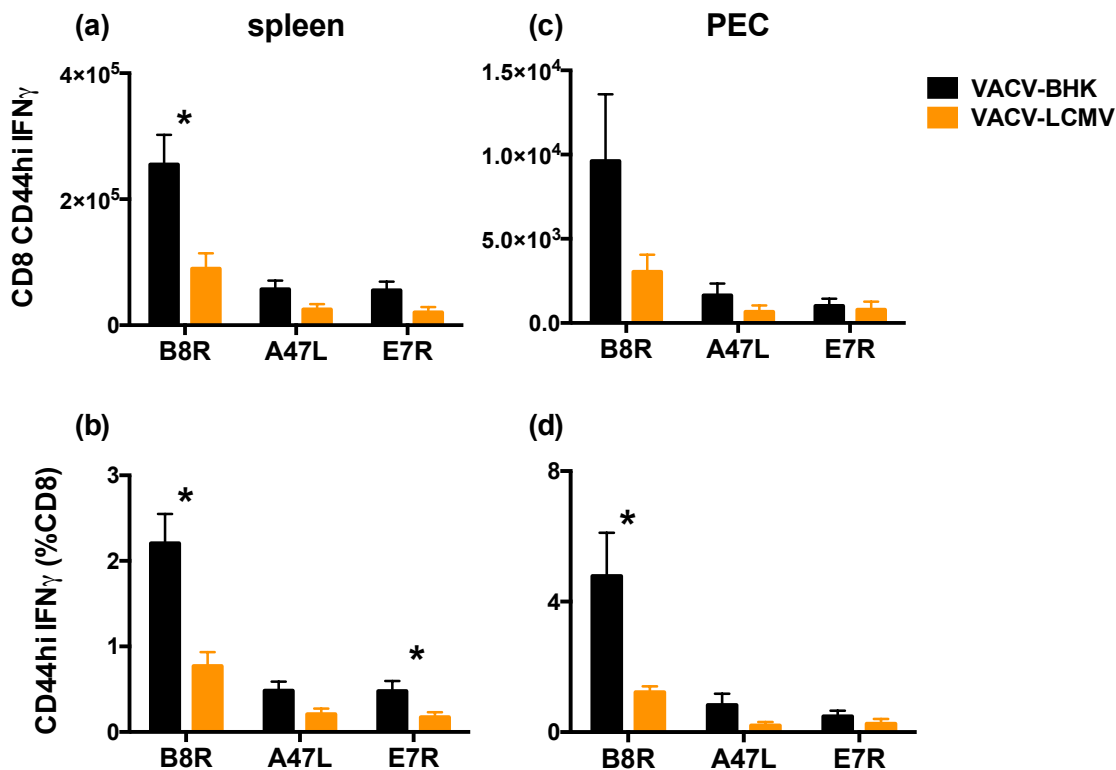


Figure 4.3. LCMV infection significantly reduces the number and frequency of VACV-specific memory CD8 T cells. VACV-immune mice were infected i.p. with 5×10^4 pfu LCMV or given supernatant from uninfected BHK culture as sham control. Memory CD8 T cells from the spleen (a and b) and peritoneum (c and d) were examined at 9 weeks post LCMV infection by ICS. (*) denotes statistical significance ($p < 0.05$) between LCMV-infected and BHK control groups (n=5).

To test whether the memory CD8 T cells that had been reduced through attrition by a heterologous infection can be further reduced by another infection with an unrelated pathogen, VACV-immune mice were given LCMV or BHK supernatant in a separate experiment. Six weeks later, the double immune mice were given MCMV or salivary gland homogenate from naïve mice. Another 6 weeks later, splenocytes were analyzed for their virus-specific memory CD8 T cells. Both LCMV and MCMV infections were found to cause a decline of pre-existing VACV B8R₂₀₋₂₇-specific memory CD8 T cell by about 50% in number and in frequency in the spleen (Fig. 4.4a and 4.4b). MCMV infection further reduced the remaining VACV-specific memory population after LCMV infection, and the percentage of B8R₂₀₋₂₇-specific CD8 T cells was significantly lower in the VACV-LCMV-MCMV triple immune mice than in the VACV-LCMV double immune group (Fig. 4.4b).

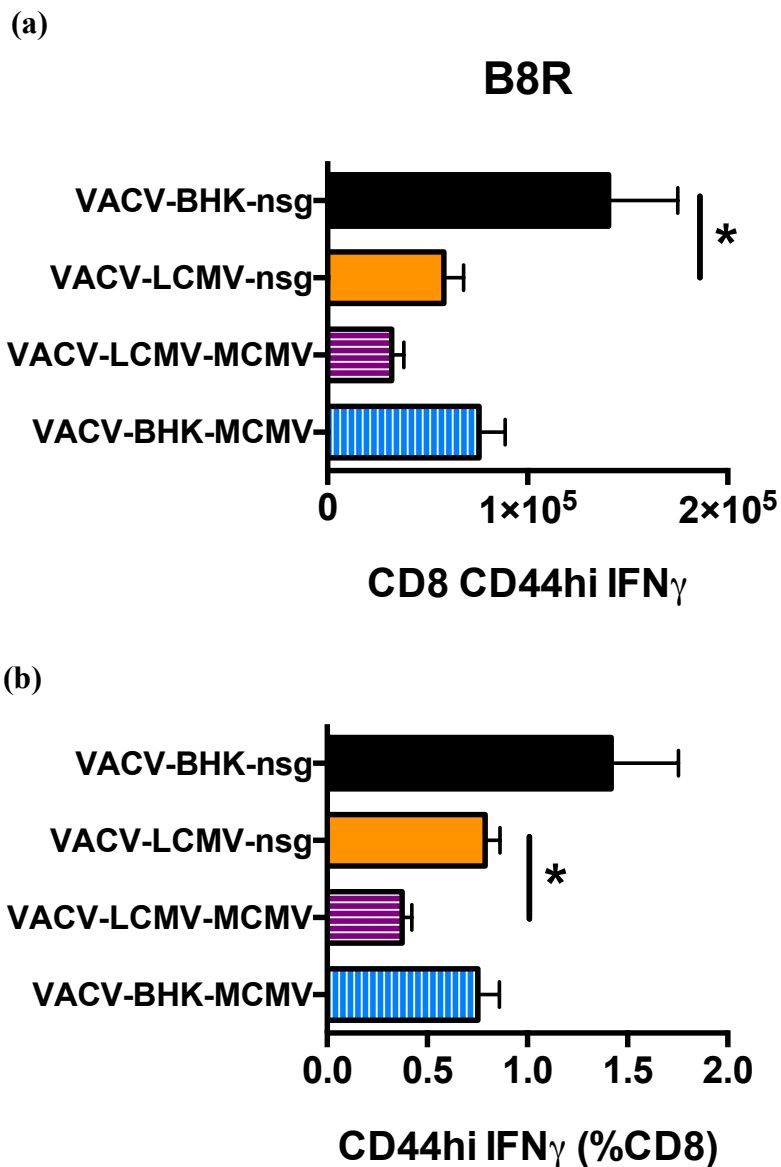


Figure 4.4. VACV B8R₂₀₋₂₇-specific memory CD8 T cell number and frequency are first reduced by LCMV infection and subsequently by MCMV infection. VACV-immune mice were infected i.p. with 5×10^4 pfu LCMV or given supernatant from uninfected BHK culture as sham control. Six weeks later, double immune mice were infected i.p. with 5×10^5 pfu MCMV or given salivary gland homogenate from naïve mice as sham control. Splenocytes were examined at 6 weeks post MCMV infection by ICS for B8R₂₀₋₂₇-specific memory CD8 T cell number (a) and frequency (b). (*) denotes statistical significance ($p < 0.05$) between groups ($n=5$).

No further erosion of LCMV-specific memory T cells by MCMV persistence

MCMV causes persistent and latent infection in mice. Re-activation of the latent virus continues to stimulate and causes sporadic proliferation of CD8 T cells, resulting in an increase of the inflationary memory T cell population. Most vigorous inflation of memory T cells happens between 8-12 weeks post MCMV infection (Snyder *et al.*, 2008). The constant activation of T cells may in theory compete with the pre-existing memory T cells for space and cytokines, leading to a passive reduction of pre-existing memory. To examine whether MCMV memory inflation can further erode pre-existing memory, LCMV-immune mice were infected with 5×10^5 pfu MCMV, and then harvested for splenocytes at different time-points to check the number and frequency of LCMV-specific memory T cells. As shown in the previous section, MCMV infection caused a reduction of both CD8 and CD4 memory T cells. However, apart from the initial attrition that happened early during infection, no further significant reduction of LCMV-specific memory CD8 T cells was found between 6-23 weeks post MCMV infection (Fig. 4.5).

CD4 memory T cells were not further eroded by MCMV persistence either (Fig. 4.6). Although memory CD4 T cell number tended to decline over time, the reduction in GP₆₁₋₈₀-specific memory CD4 T cell number was not a result of MCMV memory inflation because the decline happens after the period of time when inflation was to happen between week 8-12 post MCMV infection; and most importantly, the GP₆₁₋₈₀-specific memory CD4 T cells in both MCMV-infected and the sham control group started to decline around the same time in a similar fashion.

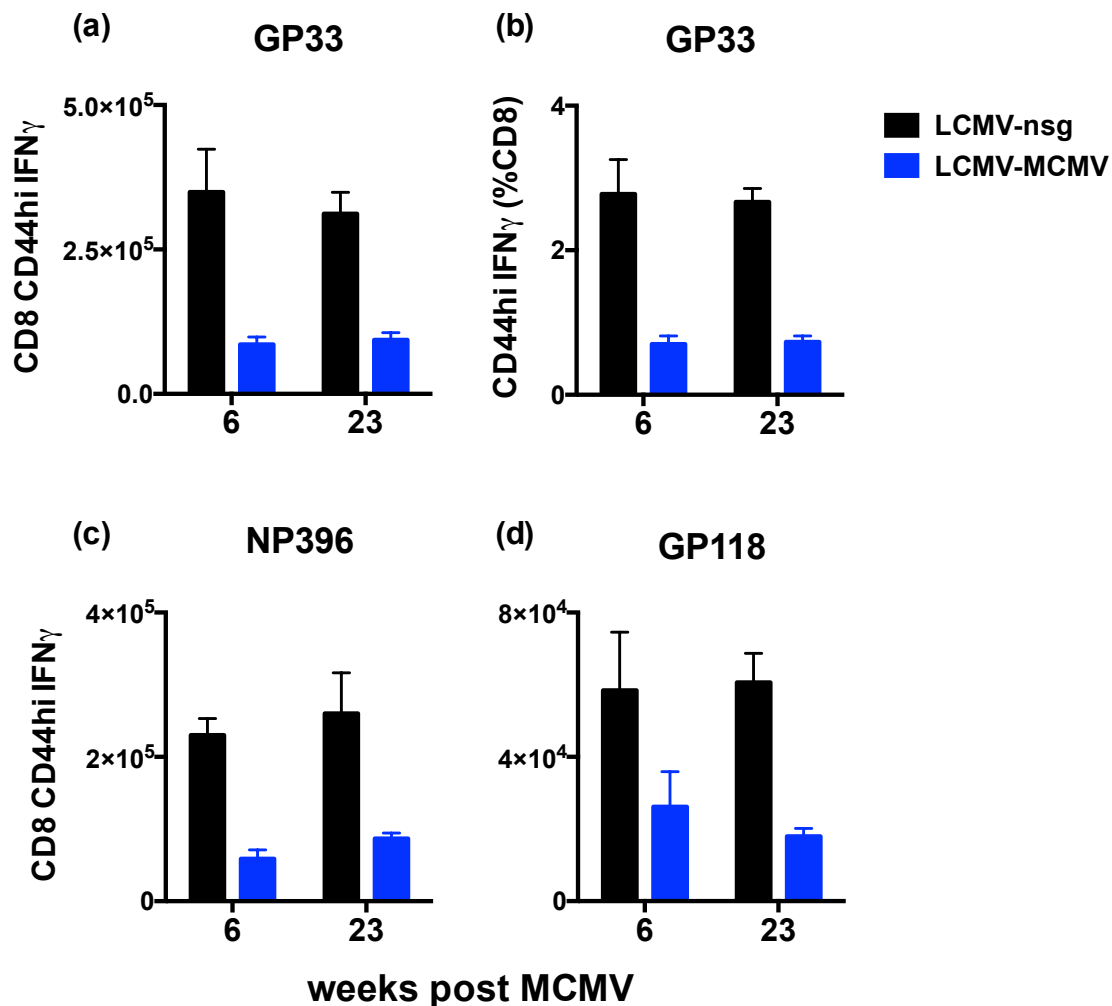


Figure 4.5. MCMV persistence does not further erode LCMV-specific memory CD8 T cells. LCMV-immune mice were infected i.p. with 5×10^5 pfu MCMV or given salivary gland homogenate from naïve mice as sham control. Memory CD8 T cells specific for (a and b) GP₃₃₋₄₁, (c) NP₃₉₆₋₄₀₄ and (d) GP₁₁₈₋₁₂₅ from the spleen were examined at week 6 and 23 post MCMV infection by ICS (n=5).

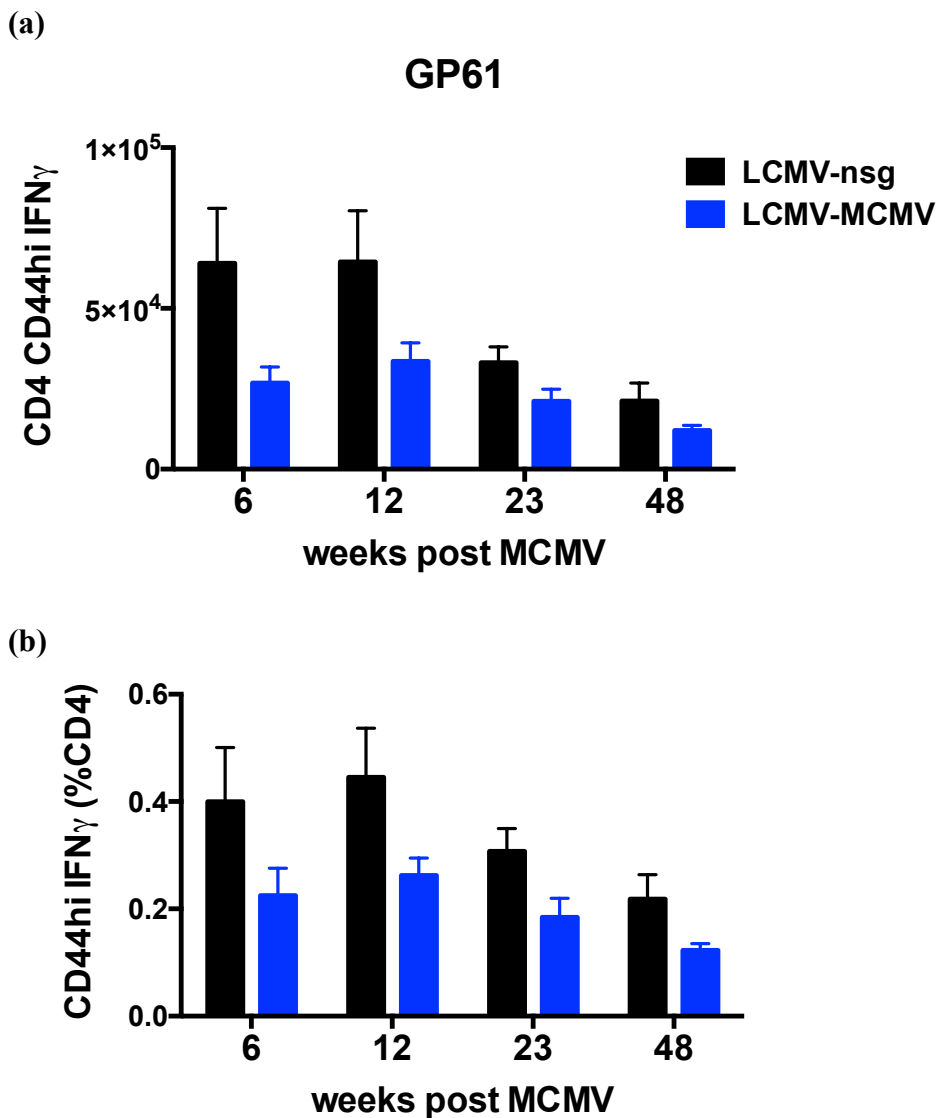


Figure 4.6. MCMV persistence does not further erode LCMV-specific memory CD4 T cells. LCMV-immune mice were infected i.p. with 5×10^5 pfu MCMV or given salivary gland homogenate from naïve mice as sham control. GP₆₁₋₈₀-specific memory CD4 T cells from the spleen were examined at week 6, 12, 23 and 48 post MCMV infection by ICS (n=5).

Increase of cross-reactive memory CD8 T cells upon heterologous infection

VACV A11R₁₉₈₋₂₀₅-specific CD8 T cells can cross-react with LCMV epitopes NP₂₀₅₋₂₁₂, GP₃₄₋₄₁ and GP₁₁₈₋₁₂₅. A11R₁₉₈₋₂₀₅-specific T cell lines grown out of LCMV-immune mice are responsive to either GP₃₄₋₄₁, GP₁₁₈₋₁₂₅ or NP₂₀₅₋₂₁₂ peptide stimulation (Cornberg *et al.*, 2010). In this system the immune response to potentially cross-reactive epitopes is highly private. Acute VACV infection selectively expands LCMV-specific memory CD8 T cells of one of the cross-reactive epitope specificities depending on the memory T cell repertoire of the individual LCMV-immune mouse (Kim *et al.*, 2005). A11R₁₉₈₋₂₀₅ is a subdominant epitope and VACV-immune mice maintained a very low number of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in the spleen (Fig. 4.7a). In the VACV-LCMV double immune mice, A11R₁₉₈₋₂₀₅-specific memory was greatly expanded in number and in percentage of CD8 T cells (Fig. 4.7a and 4.7b). The relatively large variability within groups of mice is consistent with the paradigm of private specificity. Perhaps because of the high frequency of potentially cross-reactive GP₃₄₋₄₁-, GP₁₁₈₋₁₂₅- and NP₂₀₅₋₂₁₂-specific memory CD8 T cells, LCMV-immune mice exhibited a higher number of A11R₁₉₈₋₂₀₅-responsive memory CD8 T cells than VACV-immune mice.

MCMV does not cross-react with A11R₁₉₈₋₂₀₅, and no expansion of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells occurred when VACV-immune mice were subsequently made MCMV-immune (Fig. 4.8a and 4.8b). On the other hand, the LCMV infection-boosted A11R₁₉₈₋₂₀₅-specific memory CD8 T cell number and frequency could be reduced by MCMV infection, consistent with the attrition observed in memory CD8 T cells of other specificities.

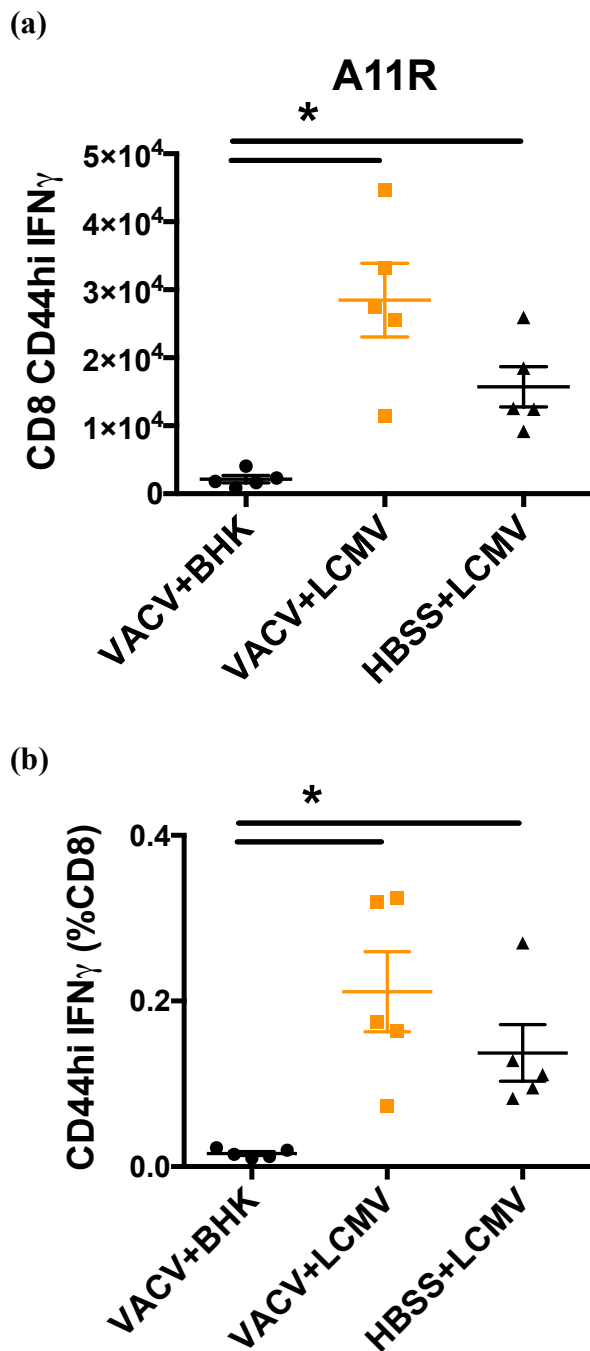


Figure 4.7. LCMV infection variably increases the cross-reactive VACV A11R₁₉₈₋₂₀₅-specific memory T cell number and frequency. VACV-immune or HBSS-injected mice were infected i.p. with 5×10^4 pfu LCMV or given supernatant from uninfected BHK culture as sham control. A11R₁₉₈₋₂₀₅-specific memory CD8 T cell number (a) and frequency (b) in the spleen were analyzed at 9 weeks post LCMV infection by ICS. (*) denotes statistical significance ($p < 0.05$) between groups.

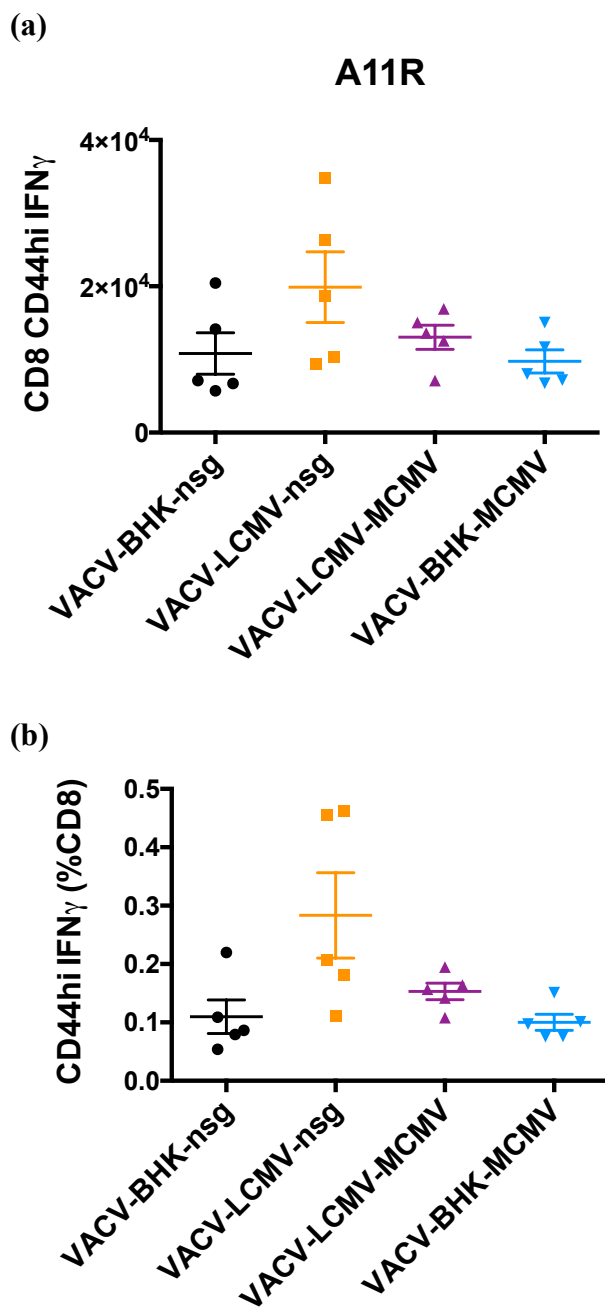


Figure 4.8. MCMV infection reduces the LCMV infection-boosted A11R₁₉₈₋₂₀₅-specific memory CD8 T cell number and frequency. VACV-immune mice were infected i.p. with 5×10^4 pfu LCMV or given supernatant from uninfected BHK culture as sham control. Six weeks later, the double immune mice were infected i.p. with 5×10^5 pfu MCMV or given salivary gland homogenate from naïve mice as sham control. A11R₁₉₈₋₂₀₅-specific memory CD8 T cell number (a) and frequency (b) in the spleen were analyzed at 6 weeks post MCMV infection by ICS.

Reduction of LCMV-specific memory CD4 T cells by VACV infection

VACV encodes decoy receptors for both type I and type II IFNs as part of its evasion strategies (Perdiguero & Esteban, 2009). The blockage may in turn deter type I IFN-induced reduction of pre-existing memory T cells. To examine the effect of VACV infection on LCMV-specific CD8 and CD4 memory T cells, LCMV-immune mice were infected i.p. with 10^6 pfu VACV or given HBSS as a control, and the splenocytes were analyzed at 8 weeks post infection. VACV infection did not reduce the examined LCMV-specific memory CD8 T cell populations (Fig. 4.9). The number and percentage of NP₃₉₆₋₄₀₄- and NP₂₀₅₋₂₁₂-specific CD8 T cells even increased in LCMV-VACV double immune mice. However, the SEM of the percentage of NP₂₀₅₋₂₁₂-specific T cells in the double immune group is relatively large because there was a 19-fold difference between the highest and the lowest frequency in the group (Fig. 4.9b). The presence of the high frequency outlier is expected because LCMV NP₂₀₅₋₂₁₂ is the most cross-reactive epitope to VACV. Despite the mild effect in the CD8 T cell compartment, VACV infection reduced the GP₆₁₋₈₀-specific memory CD4 T cells (Fig. 4.10), suggesting that VACV may also cause memory attrition in some animals.

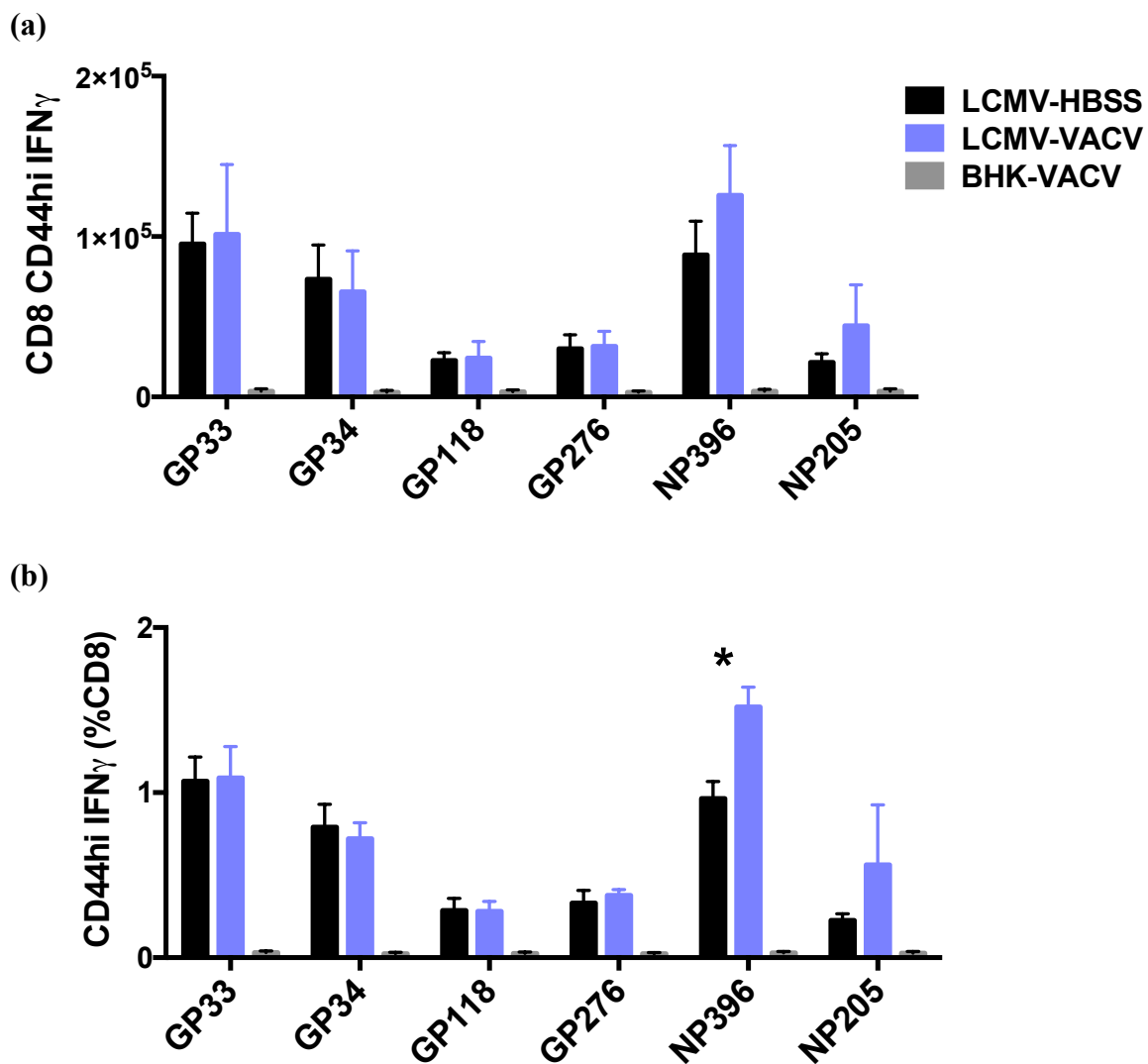


Figure 4.9. VACV infection does not reduce LCMV-specific memory CD8 T cells. LCMV-immune mice were infected i.p. with 10^6 pfu VACV or given HBSS as control. Memory CD8 T cells from the spleen were examined at 8 weeks post VACV infection by ICS. (*) denotes statistical significance ($p < 0.05$) between VACV-infected and HBSS control groups ($n=5$).

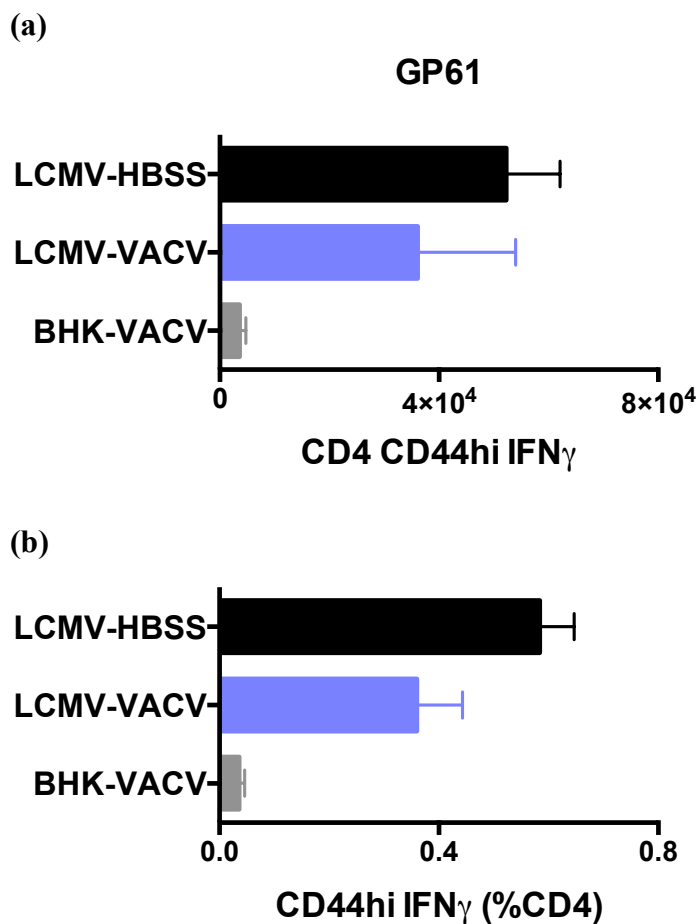


Figure 4.10. VACV infection may reduce LCMV-specific memory CD4 T cells. LCMV-immune mice were infected i.p. with 10^6 pfu VACV or given HBSS as control. GP₆₁₋₈₀-specific memory CD4 T cell number (a) and frequency (b) from the spleen were examined at 8 weeks post VACV infection by ICS.

Discussion

T cell memory can be stable and yet susceptible to changes by external influences. In this study, I showed that both CD8 and CD4 memory T cell populations could be reduced by heterologous infections with unrelated pathogens, regardless of the inherent stability of T cells. Infection with MCMV or LCMV consistently reduced pre-existing memory CD8 and CD4 T cells to previously encountered pathogens by about 50% - consistent with the published data on memory CD8 T cells (Brehm *et al.*, 2002; Kim & Welsh, 2004; Liu *et al.*, 2003; Selin *et al.*, 1996; 1999; Varga *et al.*, 2001). In contrast, VACV did not cause any decline of memory CD8 T cells of the tested specificities. This is different from previous results measured with limiting dilution assays, where VACV was able to cause a reduction of pre-existing memory CD8 T cells (Selin *et al.*, 1996). In limiting dilution assays, splenocytes are cultured for days before subjecting to cytotoxicity assay – higher IL-2-producing central memory cells are preferably retained and assayed, whereas ICS will measure every cell that produces IFN γ . It is possible that VACV infection may tend to reduce the IL-2-producing memory population but has little overall effect on the IFN γ producers. Different read-outs may result from the two assays if a good portion of the memory T cells produce IFN γ but not IL-2. Memory CD8 T cells of different phenotypes have been shown to be differentially affected by poly(I:C), which induces IFN $\alpha\beta$ and preferentially depletes memory CD8 T cells of the central memory (CD44^{hi} CD62L^{hi}) phenotype in young mice (Jiang *et al.*, 2005). Alternately, the strong heterologous immunity could have contained the infection too quickly and weaken the production of IFN $\alpha\beta$.

The recent claim that the erosion of pre-existing memory CD8 T cells by heterologous infections is only substantial in percentages (Vezys *et al.*, 2009) is insufficient to challenge the current paradigm because even in their experiment the P14 memory T cells actually underwent attrition with an end result of a statistically significant reduction in number. This fact was just overshadowed in a chart plotted with other high numbers on a linear scale. Besides, the vesicular stomatitis virus and VACV vectors used in their immunization regimen are not as strong an IFN α -inducer as LCMV is (Thompson *et al.*, 2006) and may not be as effective in actively causing memory T cell reduction.

Heterologous infection can increase the memory CD8 T cells specific for cross-reactive epitopes between two viruses. Type I IFN induces attrition of memory T cells regardless of the presence of cognate antigen early during infection (Bahl *et al.*, 2006). Nevertheless, the surviving cross-reactive memory T cells will begin to proliferate upon encounter with cognate antigen and therefore may appear unaffected by early attrition when enumerated, especially after returning to homeostasis. That was what happened with A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in some of the VACV-LCMV-immune mice and NP₂₀₅₋₂₁₂-specific memory CD8 T cells in some of the LCMV-VACV-immune mice. A similar expansion of cross-reactive NP₂₀₅₋₂₁₂-specific memory CD8 T cells has been shown in LCMV-PV and in PV-LCMV double immune mice (Brehm *et al.*, 2002), where NP₂₀₅₋₂₁₂ in LCMV and in PV differ only by the primary MHC anchoring residues at positions 5 and 8.

The influence of persistent infection on T cell memory may vary depending on the nature of the infections. Persistent LCMV clone 13 infection has been shown to cause more severe attrition of pre-existing CD8 T cell memory than acute LCMV Armstrong infection does. Not only are pre-existing memory T cells reduced early during infection, but adoptively transferred memory T cells survive poorly in the week 3-post-clone 13 host (Kim & Welsh, 2004). Persistent MCMV infection with constant activation and proliferation of inflationary memory T cells can in theory create some pressure on the memory T cell population; however, no continuous erosion of pre-existing T cell memory was found in this study during the period of memory inflation. There are at least two possible reasons for the observed differences. The cytokine environment in the MCMV-infected mice may be more supportive for homeostatic survival of memory T cells while the environment in clone 13-infected host can cause the demise of pre-existing memory T cells along with the clonal exhaustion of the clone 13-specific T cells. Besides, LCMV clone 13 persistence maintains a low level of type I IFN activity for about a month post infection (Lee *et al.*, 2009), and may promote apoptosis in memory T cells.

Reduction of memory T cells may compromise protective immunity especially when the T cell response is critical to pathogen or tumor clearance. Mice with reduced *Plasmodium berghei* circumsporozoite-specific CD8 memory T cells after a series of heterologous infections with unrelated viruses and bacteria are much less protected from *Plasmodium* sporozoites challenge than control immune mice (Schmidt & Harty, 2011), because the attrition of memory CD8 T cells has caused the number of circumsporozoite-

specific CD8 T cells to fall below the number required for sterilizing immunity (Schmidt *et al.*, 2008). Similarly, LM-OVA-immune mice infected with *Salmonella typhimurium* have a lower number of LM-OVA-specific memory T cells and are less protected from re-challenge (Dudani *et al.*, 2008). LM-OVA-immune mice infected with BCG also lose the ability to eliminate B16-OVA melanoma, although they remained protected from LM challenge (Smith *et al.*, 2002). Therefore, maintaining memory T cell number can be critical for immune protection.

Chapter V. Regulatory T cells resist virus infection-induced apoptosis

Treg cells are a heterogeneous population characterized by the expression of CD4 and the transcriptional activator and repressor Foxp3. Under the transcription control of Foxp3, Treg cells are also CD25^{hi} and CD127^{lo} (Williams & Rudensky, 2007). Expression of ICOS and TNFR2 correlates with higher suppressive activity (Chen & Oppenheim, 2011). Treg cells do not produce IL-2 or TNF (Williams & Rudensky, 2007) but depend on common γ chain cytokines from other cells for survival (Pandiyani & Lenardo, 2008). Recent studies have also suggested the involvement of TNF and type I IFN in the maintenance of Foxp3⁺ Treg cells (Chen *et al.*, 2012; Lee *et al.*, 2012).

As mentioned in Chapter IV, CD8 and CD4 T cells, particularly those of the memory phenotype, undergo apoptosis and decline in number early (day 2-4) during viral (Bahl *et al.*, 2010; Jiang *et al.*, 2003a; McNally *et al.*, 2001) or some bacterial infections (Jiang *et al.*, 2003b). This attrition is mediated in part by type I IFN, and it occurs after the treatment of mice with the type I IFN-inducer poly(I:C) (Bahl *et al.*, 2006). It can be blocked by antibody to type I IFN (Jiang *et al.*, 2005), and both attrition and apoptosis of CD44^{hi} CD8 T cells are dramatically reduced in type I IFN receptor (IFNAR) KO mice (Bahl *et al.*, 2006; McNally *et al.*, 2001). The CD44^{hi} memory CD8 and CD4 T cells show the highest levels of apoptotic loss at these early stages of infection, perhaps making room for a new immune response to develop rapidly (Bahl *et al.*, 2006).

Infection has been implicated as a potential trigger in autoimmune diseases (Huber, 2006; Sherbet, 2009; Van Der Werf *et al.*, 2007; Zipris *et al.*, 2003), but the underlying mechanism has yet to be illuminated. One of the theories is that infection

may disrupt the balance of immune regulation (perhaps through Treg cells) at the susceptible organs (Jun & Yoon, 2003; Zipris *et al.*, 2003). Foxp3⁺ CD4⁺ Treg cells exhibit a partial memory phenotype with an intermediate to high expression of CD44 (Min *et al.*, 2007). Therefore, I questioned whether Treg cells might behave like the CD44^{hi} CD4 and CD8 T cells and be driven into apoptosis, causing a reduction in cell number during early stages of infection. If so, their demise might contribute to the phenomenon of virus-induced autoimmunity.

Results

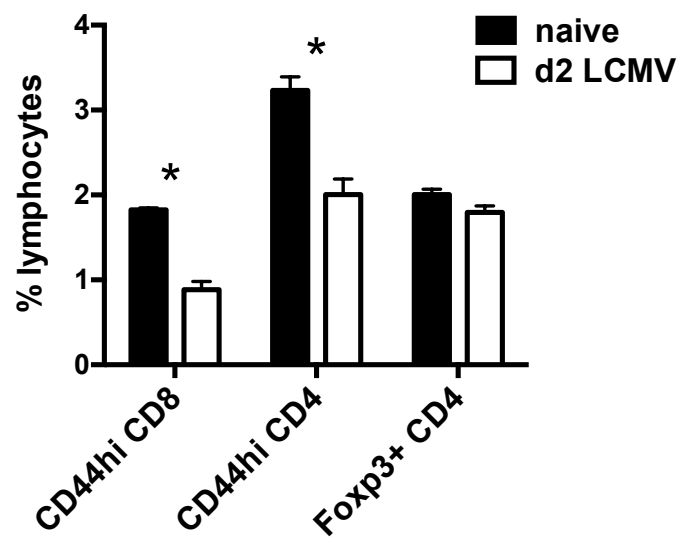
Insignificant reduction of Foxp3⁺ CD4⁺ Treg cells after LCMV infection

To investigate whether Foxp3⁺ Treg cells undergo attrition like memory T cells do early after LCMV infection, Foxp3GFP knock-in mice were inoculated with 5×10^4 pfu LCMV. LCMV induces type I IFN that peaks at day 2 post infection (Welsh, 1978). Splenocytes from infected and aged-matched naïve control mice were harvested two days post infection and analyzed by flow cytometry. While the frequency of CD44^{hi} CD4 and CD44^{hi} CD8 T cells among lymphocytes decreased significantly after LCMV infection, the frequency of Foxp3⁺ CD4 T cells remained the same (Fig. 5.1a). The actual number of T cells in the spleen was then calculated for each individual mouse using their total splenocyte counts separately. As expected, CD44^{hi} CD4 and CD44^{hi} CD8 T cells declined significantly in number (Fig. 5.1b). However, the total number of Foxp3⁺ CD4 T cells did not significantly change after infection (Fig. 5.1b). At day 3 post LCMV infection, a small but consistent decline in the frequency of Foxp3⁺ CD4 T cells was observed in the spleen (Fig. 5.1c), but the actual number of Foxp3⁺ CD4 T cells in the spleen of infected animals was not significantly different from that of naïve animals (Fig. 5.1d). Therefore, the presence of Foxp3⁺ CD4 T cells in the spleen is relatively stable early during LCMV infection.

Infection can cause Foxp3⁺ CD4 T cells to traffic between the secondary lymphoid organs. Preliminary data suggested that at day 2 post infection while the number of Treg cells slightly decreased in inguinal lymph nodes, the number in mesenteric lymph nodes increased. The number of Treg cells did not change in

axillary/brachial lymph nodes in the first two days but slightly increased on day 3 post infection when the number of Treg cells started to return to the level before infection in the inguinal lymph nodes. Overall, no significant change in the number and frequency of Treg cells was found in the lymph nodes examined. Due to the variability in cell counts, more experiments are needed to confirm the observation regarding the number of Treg cells in the lymph nodes.

(a)



(b)

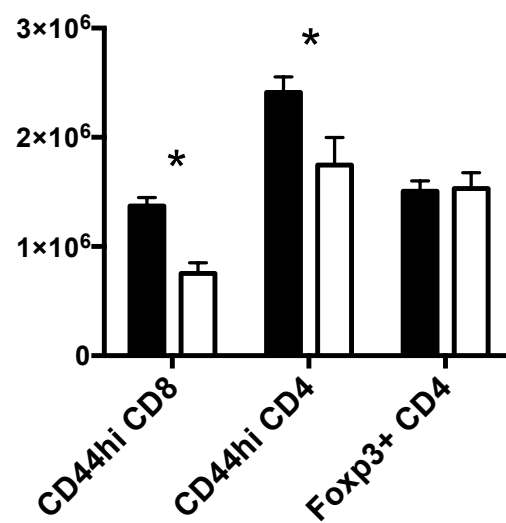
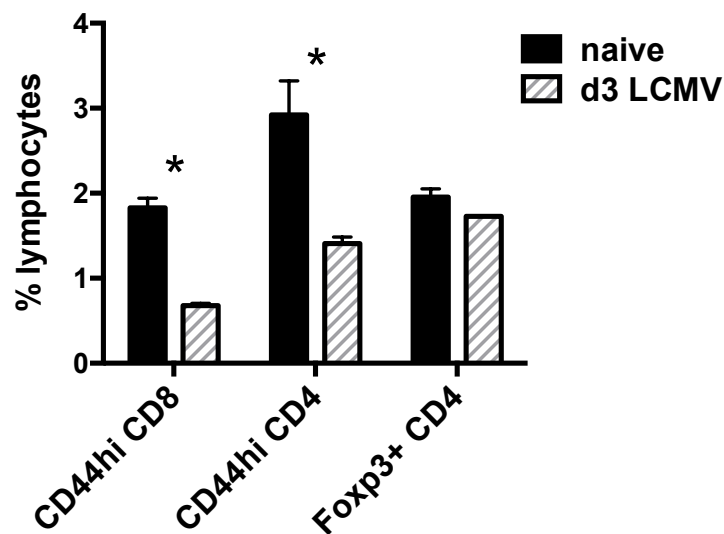


Figure 5.1.

(c)



(d)

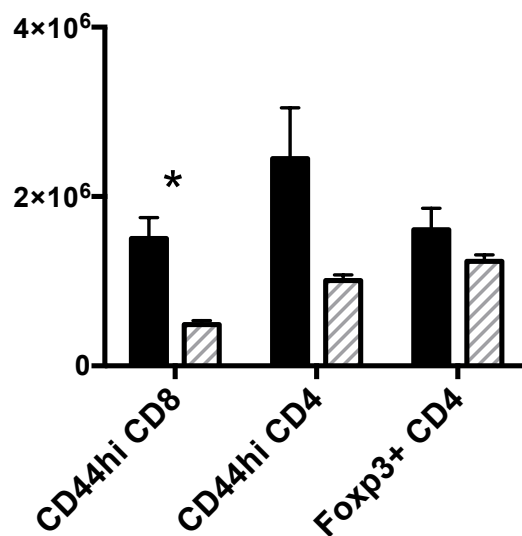


Figure 5.1. Foxp3+ CD4+ Treg cells resist loss after LCMV infection. Foxp3GFP mice were infected i.p. with 5×10^4 pfu LCMV. Two (a and b) or three (c and d) days later, surface stained splenocytes from infected and uninfected (naïve) mice were analyzed by flow cytometry. The frequency (a and c) and number (b and d) of CD44hi CD4 and CD44hi CD8 T cells were significantly reduced in the spleen after infection, while there was no significant reduction of Foxp3+ CD4+ Treg cells. (a and b) Combined data from 3 separate experiments ($n=9$). (c and d) Representative data of seven separate experiments ($n=3$). (*) denotes statistical significance ($p < 0.05$) between naïve and LCMV-infected groups.

Foxp3+ CD4 T cells are more resistant to apoptosis during LCMV infection

Cell death by apoptosis is a coordinated process. At the end stage of apoptosis, activated caspase-3 cleaves the inhibitors of DNA fragmentation factor 45 (DFF45) and DFF35, releasing active nuclease DFF40 (caspase-activated DNase) into the nucleus (Gu *et al.*, 1999), and cleaves and inactivates poly-(ADP-ribose)-polymerase (PARP), which functions in DNA single-strand break repair (Fink & Cookson, 2005). Both events ultimately lead to DNA fragmentation. The 3'-OH end of the fragmented or nicked DNA can be visualized by TUNEL assay, which covalently attaches a dUTP to the 3'-OH end. Subsequent labeling with a fluorescence-conjugated anti-dUTP antibody allows quantitative measurement of DNA fragmentation by flow cytometry. A previous study showed that both CD44hi CD8 and CD44hi CD4 T cells in the spleen were more TUNEL positive after poly (I:C) treatment (Bahl *et al.*, 2010). Infection with LCMV also increased the frequency of TUNEL positive CD44hi CD8 T cells in the spleen early after infection (Bahl *et al.*, 2010), and tissue sections have shown two peaks of TUNEL positive splenocytes after LCMV infection: one at day 3, closely following the peak of the IFN response, which I examined here, and the other at day 11, as the response enters the contraction phase (Razvi *et al.*, 1995).

To test if Treg cells are also sensitive to virus infection-induced apoptosis, splenocytes were harvested from Foxp3GFP mice at day 2 post LCMV infection, rested in media at 37°C for 5 hrs and stained with the TUNEL assay. Without infection, CD44hi CD4 and Foxp3+ CD4 T cells appeared to be more TUNEL positive than CD8 T cells (Fig. 5.2a). Infection with LCMV increased the percentage of TUNEL positive

CD4 and CD8 T cells but had no effect on Foxp3⁺ CD4 T cells (Fig. 5.2). As expected, the increase in apoptotic events among CD44^{hi} T cells was more dramatic than that among CD44^{lo} T cells after LCMV infection (Fig. 5.2b). On the other hand, Foxp3⁺ CD4 T cells were slightly apoptotic before and after infection with LCMV regardless of the status of CD44 expression. Although Foxp3⁺ CD4 T cells from naïve mice had a measurable TUNEL background, they were as apoptotic as the relatively resistant CD44^{lo} CD4 and CD44^{lo} CD8 T cells at day 2 post LCMV infection, and they were much more resistant to apoptosis than were CD44^{hi} CD4 and CD44^{hi} CD8 T cells.

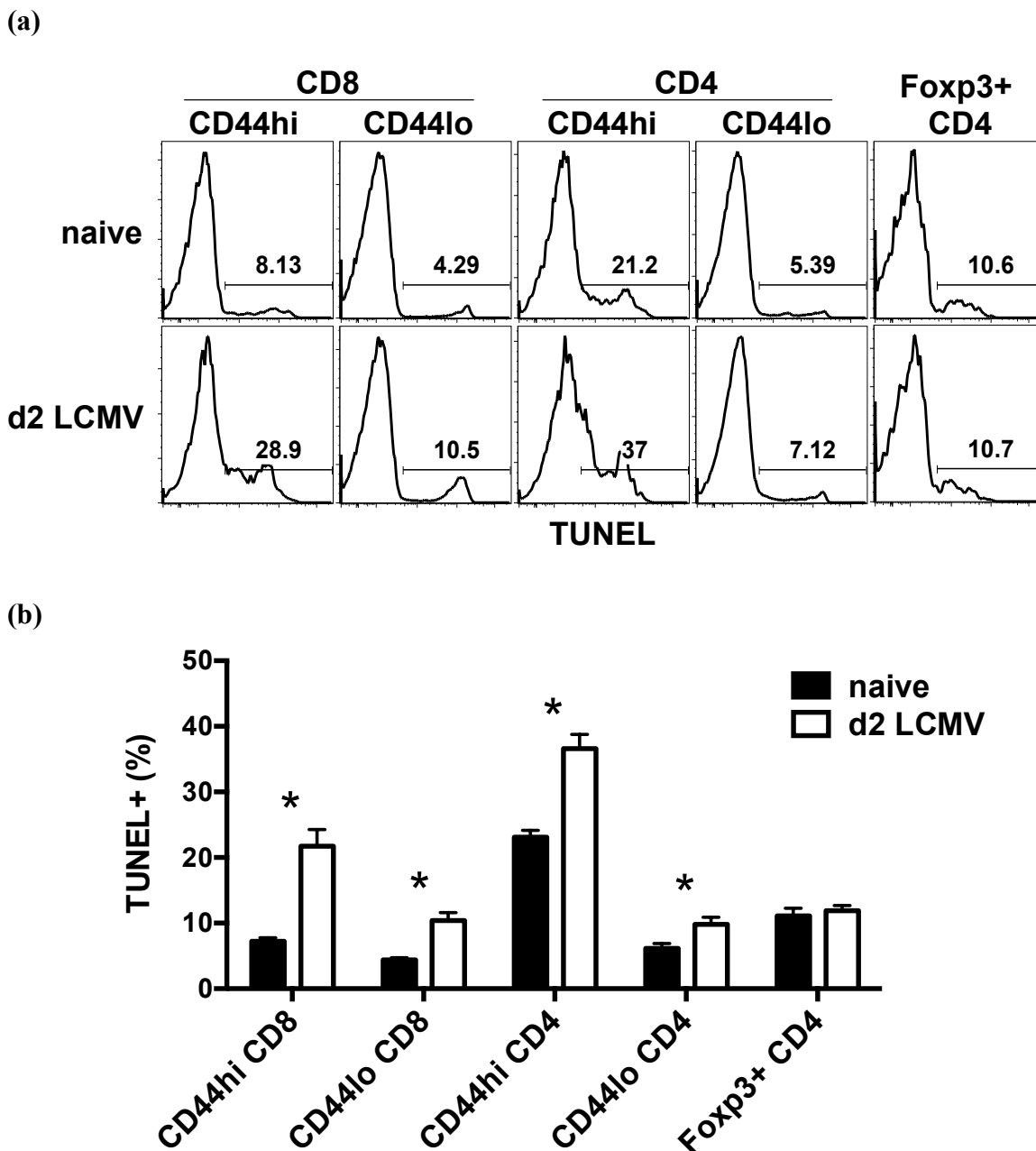


Figure 5.2. Foxp3⁺ CD4⁺ Treg cells are less TUNEL positive than CD44^{hi} CD4 and CD44^{hi} CD8 T cells in the spleen at day 2 post LCMV infection. Foxp3GFP mice were infected i.p. with 5×10^4 pfu LCMV. Splenocytes harvested at 2 days post infection were stained with TUNEL after incubating in media at 37°C for 5 hr. (a) Histograms of TUNEL staining intensity of CD4, CD8 and Treg cells from a naïve and a day 2 LCMV-infected animal in a representative experiment. (b) Combined data from 3 separate experiments (total n = 9). (*) denotes statistical significance ($p < 0.05$) between naïve and day 2 post LCMV infection.

Activated caspase disturbs the asymmetry of the plasma membrane, causing exposure of phosphatidylserine on the cell surface (Fink & Cookson, 2005). Therefore, apoptosis of splenocytes from LCMV-infected Foxp3GFP mice was also examined *ex vivo* with amine-reactive live/dead stain and Annexin V, which binds to exposed phosphatidylserine. Similar to data from the TUNEL assay, CD44^{hi} CD4 T cells were highly Annexin V positive even among cells from naïve spleens (Fig. 5.3). Apoptotic events among CD4 and CD8 T cells increased after LCMV infection but remained low among Foxp3⁺ CD4⁺ Treg cells (Fig. 5.3).

At day 3 post LCMV infection, although Foxp3⁺ CD4 T cells from naïve animals displayed some TUNEL and Annexin V background staining, their staining intensity was not increased by LCMV infection, while CD8 and CD4 T cells, especially the CD44^{hi} subsets, became much more TUNEL and Annexin V positive after infection (Fig. 5.4). Both the TUNEL and Annexin V data suggest that Foxp3⁺ CD4⁺ Treg cells are much more resistant to virus infection-induced apoptosis than CD44^{hi} CD4 and CD44^{hi} CD8 T cells in the spleen.

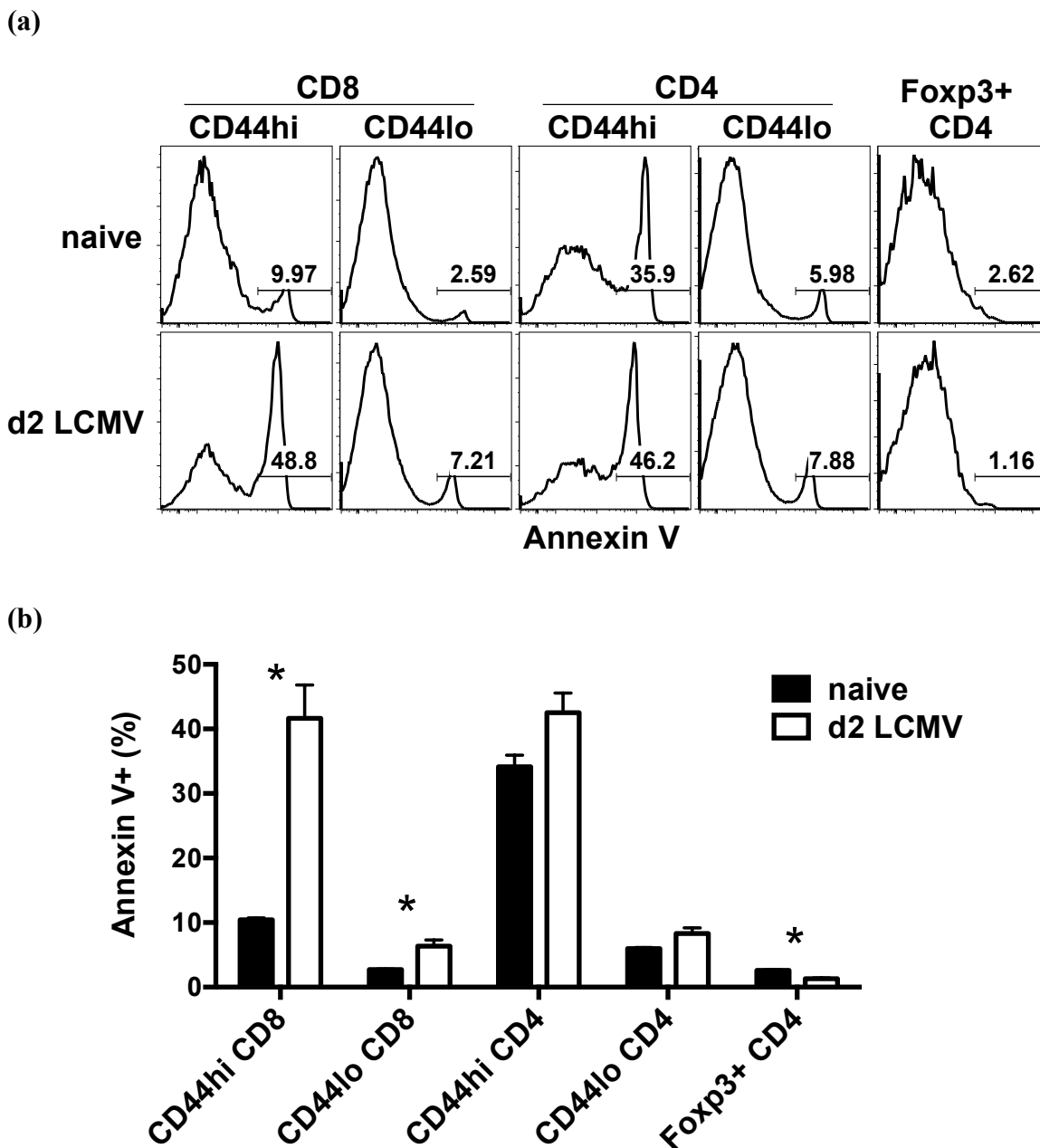


Figure 5.3. Foxp3⁺ CD4⁺ Treg cells are less Annexin V positive than CD8 and CD4 T cells in the spleen at day 2 post LCMV infection. Foxp3GFP mice were infected i.p. with 5×10^4 pfu LCMV. Splenocytes harvested at 2 days post infection were stained *ex vivo* with live/dead fixable stain and Annexin V. (a) Histograms of Annexin V staining intensity of live CD4, CD8 and Treg cells from a naïve and a day 2 LCMV-infected animal in a representative experiment. (b) Data from a representative of three separate experiments (n=3). (*) denotes statistical significance ($p < 0.05$) between naïve and day 2 post LCMV infection.

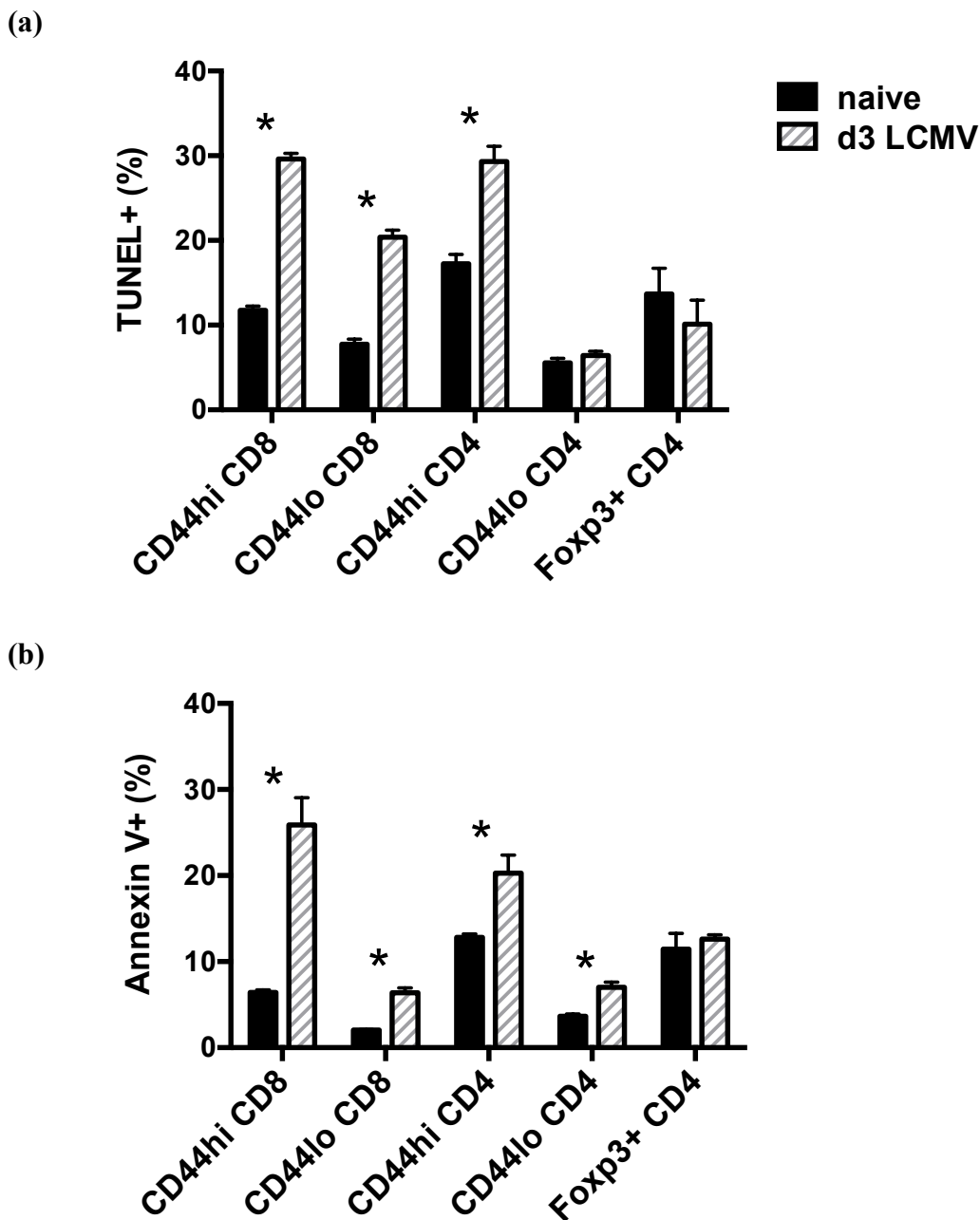


Figure 5.4. Foxp3⁺ CD4⁺ Treg cells are less apoptotic than CD44^{hi} CD4 and CD8 T cells in spleen at day 3 post LCMV infection. Foxp3GFP mice were infected i.p. with 5×10^4 pfu LCMV. Splenocytes harvested at 3 days post infection were stained (a) with TUNEL after incubating in media at 37°C for 5 hrs or (b) *ex vivo* with live/dead fixable stain and Annexin V. Data are representative of at least three separate experiments (n = 3). (*) denotes statistical significance ($p < 0.05$) between naïve and day 3 post LCMV infection.

Differential regulation of receptors for survival signals during LCMV infection

With the observed differences in sensitivity to virus infection-induced apoptosis, I questioned what might contribute to the survival advantage of Foxp3⁺ CD4⁺ Treg cells at the early stage of infections. IL-7 signaling is critical for the survival of naïve (Boursalian & Bottomly, 1999; Tan *et al.*, 2001) and memory T cells (Kondrack *et al.*, 2003; Schluns *et al.*, 2000) in the periphery, whereas IL-2 is important for Treg cell maintenance (Setoguchi *et al.*, 2005). Therefore, I checked the surface expression of IL-7 receptor α (CD127), IL-2 receptor α (CD25), β (CD122) and the common cytokine receptor γ chain (CD132) on T cells in naïve and LCMV-infected Foxp3GFP mice at day 2 post infection. As published previously (Simonetta *et al.*, 2010), surface expression of CD127 on Foxp3⁺ CD4 T cells was much lower than that on Foxp3 negative CD4 T cells in the spleen. CD127 expression on CD44^{hi} CD4 T cells was lower than that on CD44^{hi} CD8 T cells (Fig. 5.5a). After LCMV infection, CD127 was down-regulated in all CD8 and CD4 T cells regardless of Foxp3 or CD44 expression status. Expression of CD25 was high on Foxp3⁺ CD4⁺ Treg cells but was negligible on all other CD4 and CD8 T cells (Fig. 5.5b). LCMV infection up-regulated CD25 expression on Treg cells significantly but did not affect expression on other CD4 or CD8 T cells at day 2 post infection. As previously published (Zhang *et al.*, 1998), the expression of CD122 was the highest on CD44^{hi} CD8 T cells but very low on CD44^{lo} CD4 T cells. Foxp3⁺ CD4⁺ Treg cells expressed higher levels of CD122 than CD44^{hi} CD4 and CD44^{lo} CD8 T cells (Fig. 5.5c). CD122 expression was not altered by LCMV infection. All CD4 and CD8 T cells and Treg cells expressed CD132 (Fig. 5.5d). Although the expression of CD132

was elevated on CD4 and CD8 T cells after LCMV infection, it was unchanged on Treg cells. The pattern of receptor expression indicates that although LCMV infection down-regulates IL-7 receptor on all T cells, the high-affinity IL-2 receptor remains intact on Treg cells; this may provide a survival advantage to Treg cells.

The IL-15 receptor shares CD122 and CD132 with the IL-2 receptor. IL-15 stimulates proliferation of CD44^{hi} CD122^{hi} CD8 T cells *in vitro* (Zhang *et al.*, 1998) and IL-15 receptor α KO mice are unable to maintain memory CD8 T cells long term (Becker *et al.*, 2002; Burkett *et al.*, 2003). IL-15 is needed for CD44^{hi} CD8 T cells to recover in number after an apoptotic depletion by type I IFN (Kim & Welsh, 2004). The presence of IL-15 receptor α (CD215) was very rare on T cells in naïve animals but increased substantially especially among CD44^{hi} CD8 T cells after LCMV infection (Fig. 5.5e).

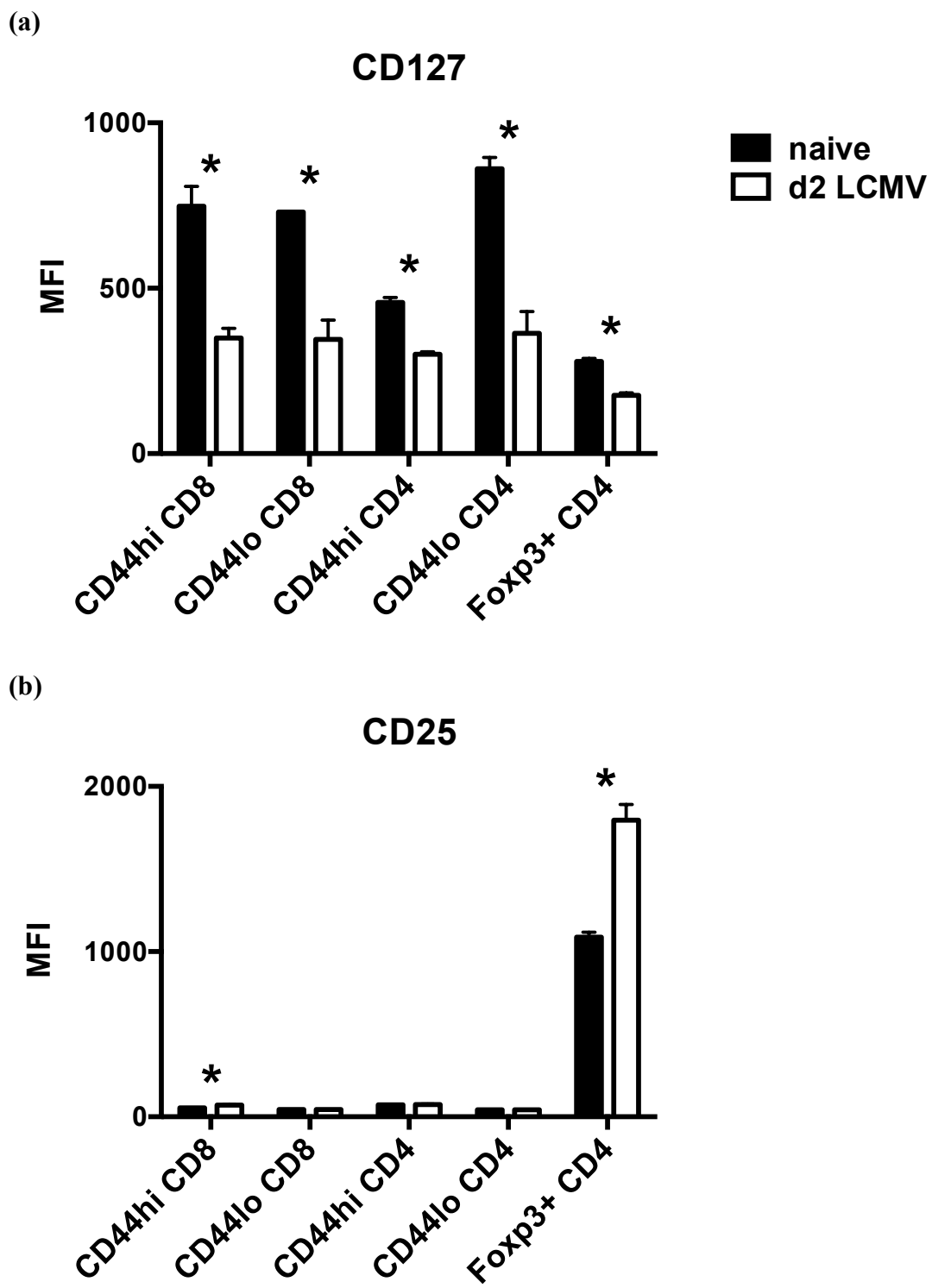


Figure 5.5.

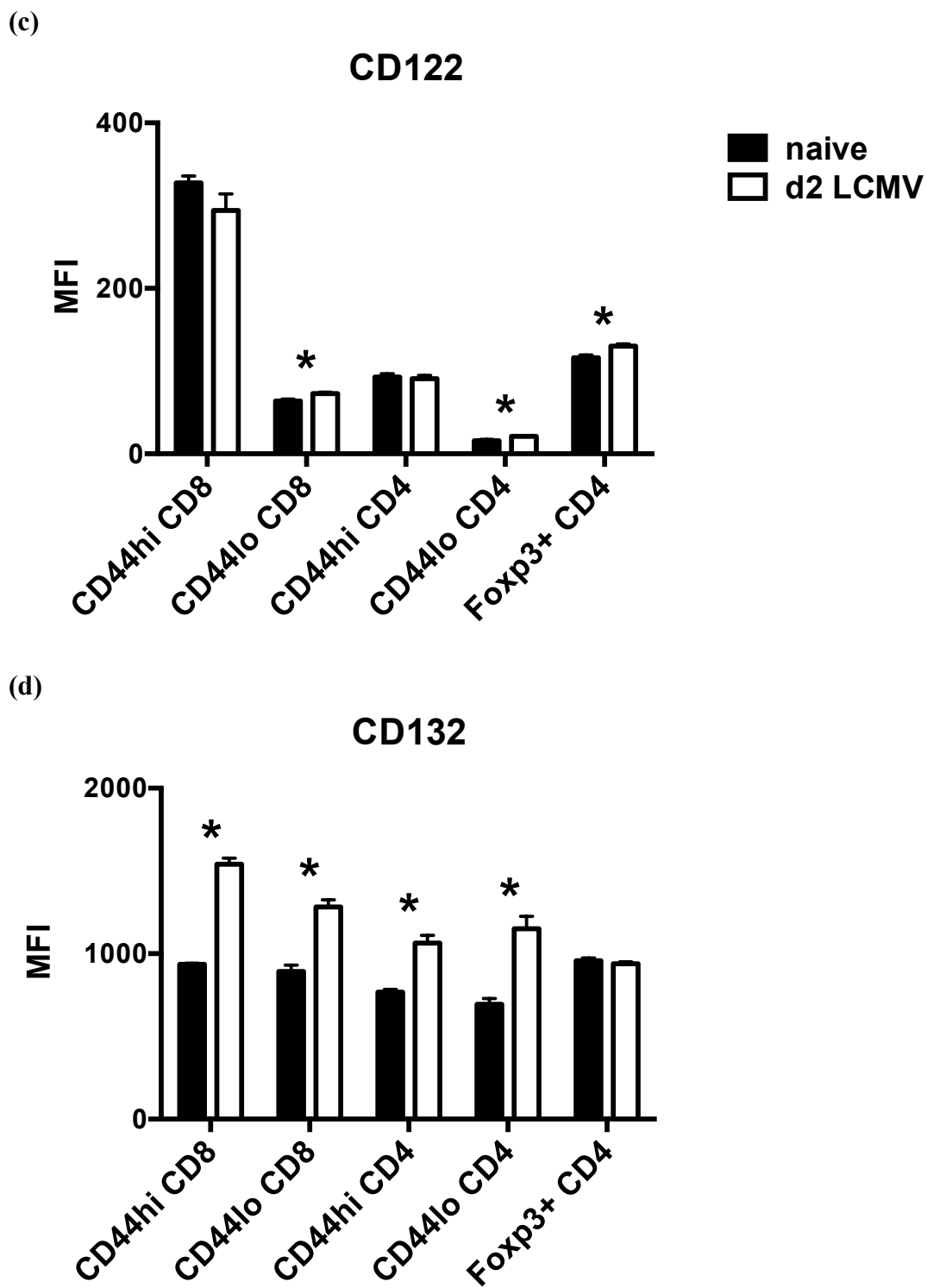


Figure 5.5.

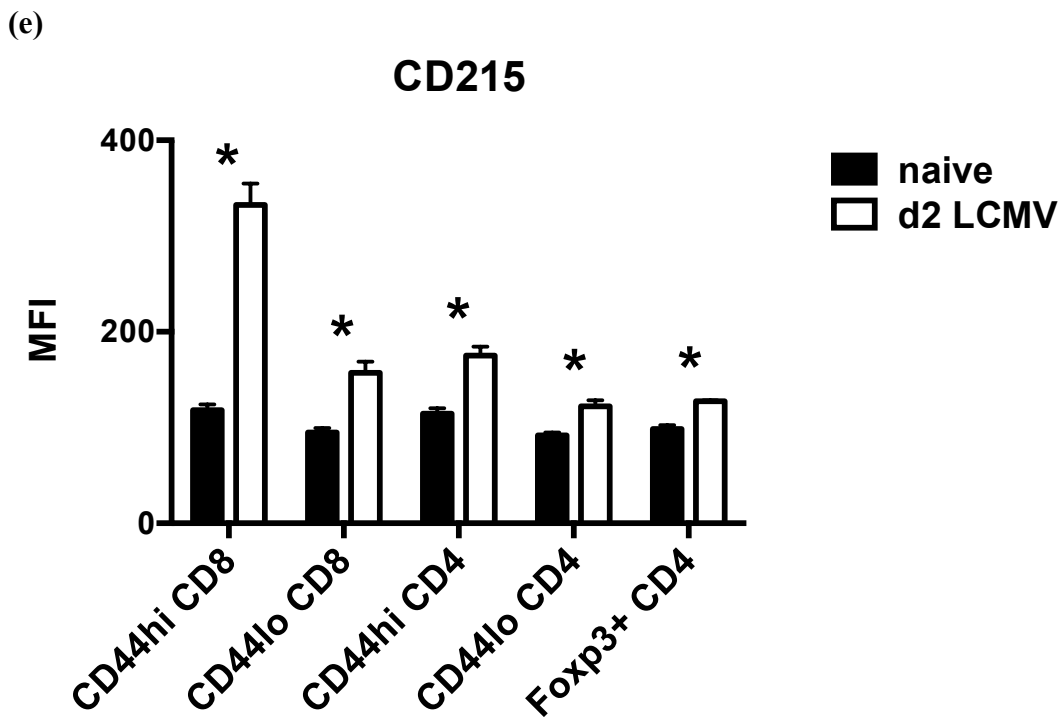


Figure 5.5. IL-7 receptor is down-regulated on T cells while high-affinity IL-2 receptor remains on Foxp3⁺ CD4⁺ Treg cells after LCMV infection. Foxp3GFP mice were infected i.p. with 5×10^4 pfu LCMV. Splenocytes harvested at 2 days post infection were stained *ex vivo* for CD127 (a), CD25 (b), CD122 (c), CD132 (d) and CD215 (e). Geometric mean fluorescence intensity data are representative of at least three separate experiments. (*) denotes statistical significance ($p < 0.05$) between naïve and day 2 post LCMV-infected mice ($n=3$).

STAT5A and STAT5B mediate IL-2, IL-7 and IL-15 signaling (Tripathi *et al.*, 2010; Wuest *et al.*, 2008; Xue *et al.*, 2002). Phosphorylated STAT5 is involved not only in sustaining Foxp3 expression (Burchill *et al.*, 2008), but also in regulating CD8 T cell homeostasis (Hand *et al.*, 2010; Kelly *et al.*, 2003). When the expression of STAT5 before and after LCMV infection was compared, I found that the total expression of STAT5 was the highest among Foxp3⁺ CD4 T cells in naïve animals and was further increased after LCMV infection (Fig. 5.6a). In contrast, the total STAT5 expression was slightly reduced among CD8 T cells (both CD44^{hi} and lo) and CD44^{lo} CD4 T cells after infection. Consistent with the expression pattern of CD25 (Fig. 5.5b), only Treg cells were able to respond to IL-2 stimulation *in vitro* and phosphorylate STAT5 (Fig. 5.6b). Time course experiments also revealed that STAT5 in Foxp3⁺ CD4 T cells could be phosphorylated in response to IL-2 instantaneously without any incubation (data not shown). Therefore, Foxp3⁺ CD4⁺ Treg cells were able to receive survival signals through IL-2 receptor and STAT5 early during LCMV infection.

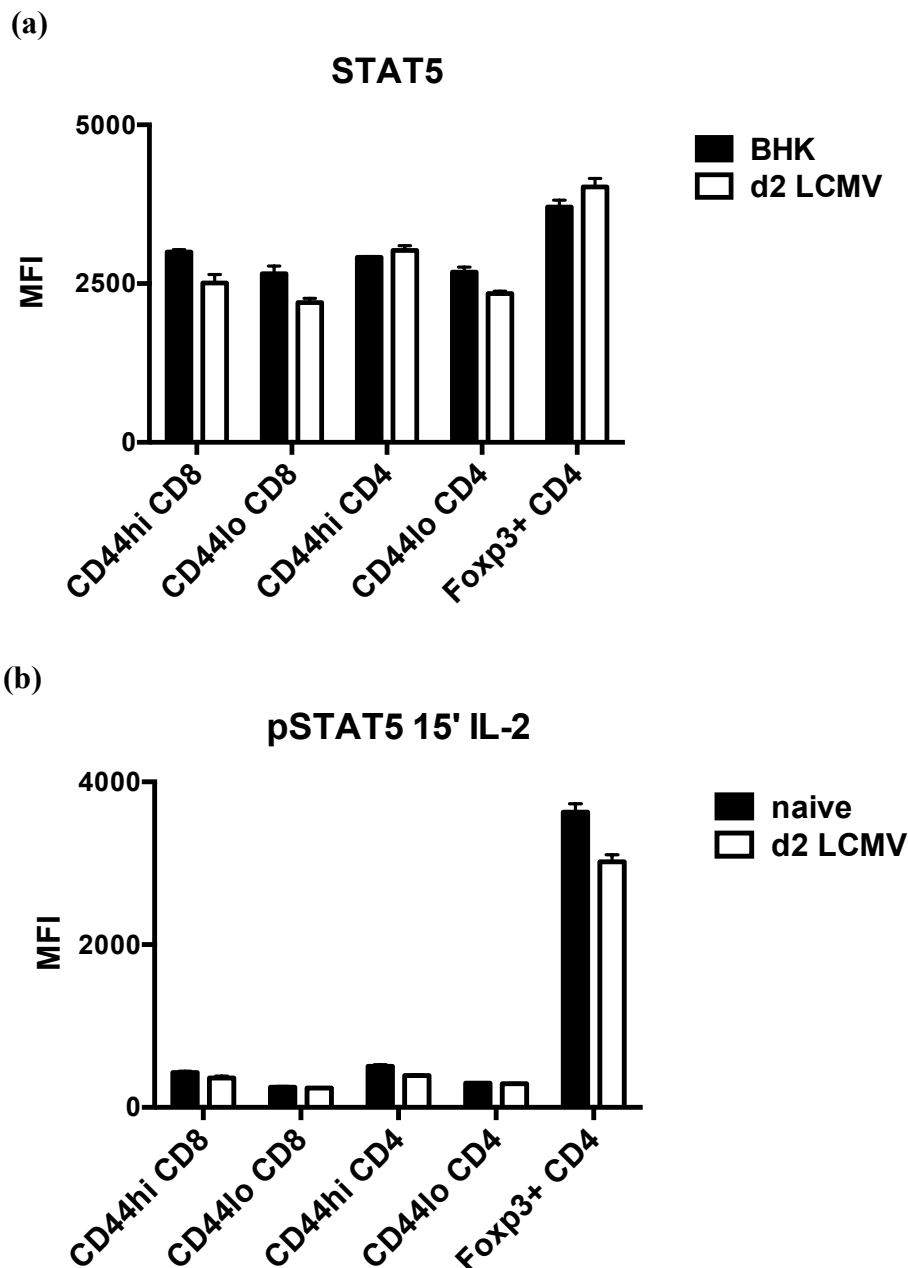


Figure 5.6. Foxp3⁺ CD4⁺ Treg cells display the highest level of Stat5 expression and respond well to IL-2 stimulation *in vitro* even after LCMV infection. Foxp3GFP mice were infected i.p. with 5×10^4 pfu LCMV. (a) Splenocytes harvested at 2 days post infection were stained *ex vivo* for STAT5. (b) Phosphorylation of STAT5 was assessed after stimulating splenocytes with 5 ng/ml IL-2 for 15 minutes at 37°C. Data are representative of two separate experiments showing geometric mean fluorescence intensity between BHK sham control or naïve versus day 2 post LCMV-infected groups of 2-3 mice.

Balance between pro- and anti-apoptotic molecules altered by LCMV infection

BH3-only Bim is involved in regulating survival of T cells including Treg cells (Chougnnet *et al.*, 2011; Wojciechowski *et al.*, 2007). LCMV-induced apoptosis of CD44^{hi} CD8 T cells involves Bim (Bahl *et al.*, 2010). To initiate apoptosis, Bim antagonizes the anti-apoptotic molecule Bcl-2 and its family members such as Bcl-xL and myeloid cell leukemia sequence 1 (Mcl-1), which would otherwise prevent the effectors Bcl-2-associated X protein (Bax) and Bcl-2 antagonist or killer (Bak) from forming pores on the mitochondrial outer membrane (Youle & Strasser, 2008). The balance between pro-apoptotic and anti-apoptotic molecules can influence cell survival. To see if LCMV infection would shift this balance, I examined the geometric mean fluorescence intensity (MFI) ratio between these molecules. C57BL/6 mice were used in place of FoxpGFP mice because of the sensitivity of the Bcl-2 epitope to methanol treatment and because the green fluorescent protein could not survive the 1-hr saponin permeabilization. At day 2 post LCMV infection, there was a decrease in Bcl-2 in CD44^{hi} CD8 T cells (Fig. 5.7a). Since the relative abundance of Bim among different T cell populations (Fig. 5.7b) was very similar to that of Bcl-2, the Bim:Bcl-2 MFI ratio was similar across different T cell populations (Fig. 5.7c). After infection, there was a slight increase of the Bim:Bcl-2 MFI ratio across all T cells. On the other hand, LCMV infection slightly increased the expression of Bcl-xL (Fig. 5.7d) and reduced the Bim:Bcl-xL MFI ratio in CD8 T cells and CD44^{lo} CD4 T cells (Fig. 5.7e). The expression of Mcl-1 was increased in all T cells after LCMV infection (Fig. 5.7f) and reduced the Bim:Mcl-1 MFI ratio (Fig. 5.7g).

Bak on the mitochondria does not interact with Bim directly (Willis *et al.*, 2007) but is restrained by Mcl-1 and Bcl-xL (Willis *et al.*, 2005). The N-terminus becomes exposed on activated Bak, which homo-oligomerizes to form pores on the outer membrane of mitochondria (Tait & Green, 2010). The level of the active form of Bak was much higher in CD8 T cells than in CD4 T cells and was slightly increased in CD44^{hi} CD8 T cells, CD4 T cells and Treg cells after LCMV infection (Fig. 5.7h). Because of the increased Mcl-1 expression (Fig. 5.7f), the active Bak:Mcl-1 MFI ratio was reduced in CD8 T cells but was not changed in CD4 T cells after infection (Fig. 5.7i). The active Bak:Bcl-xL MFI ratio was the highest in CD44^{lo} CD8 T cells and was decreased after LCMV infection (Fig. 5.7j). LCMV infection altered the ratio between pro- and anti-apoptotic molecules, but so far no correlation can be established between the expression ratios and the differential susceptibilities of the five T cell populations to infection-induced apoptosis. Further examination on the other molecules in the cell death pathways may be needed to decipher the mechanism(s) involved.

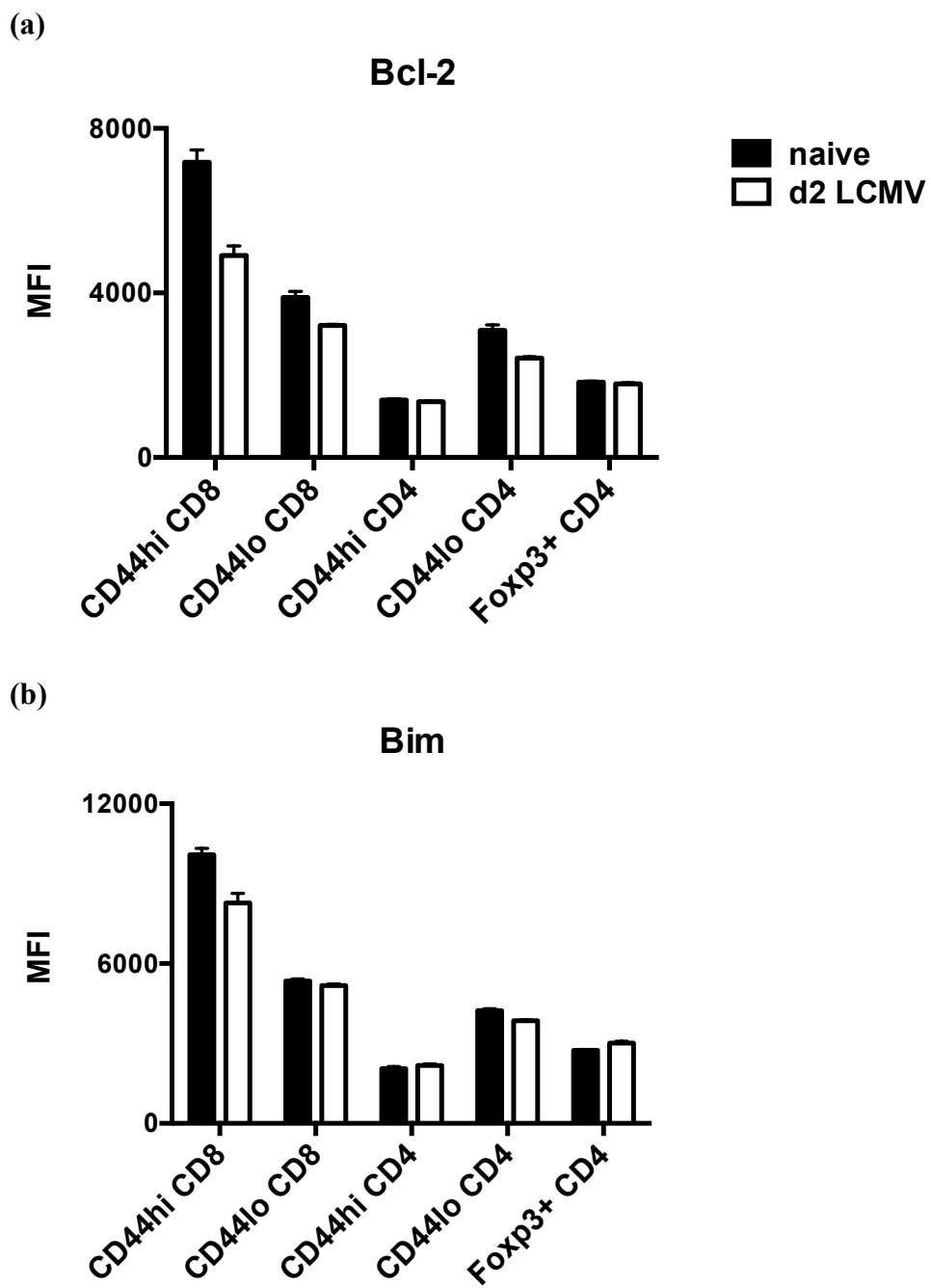


Figure 5.7.

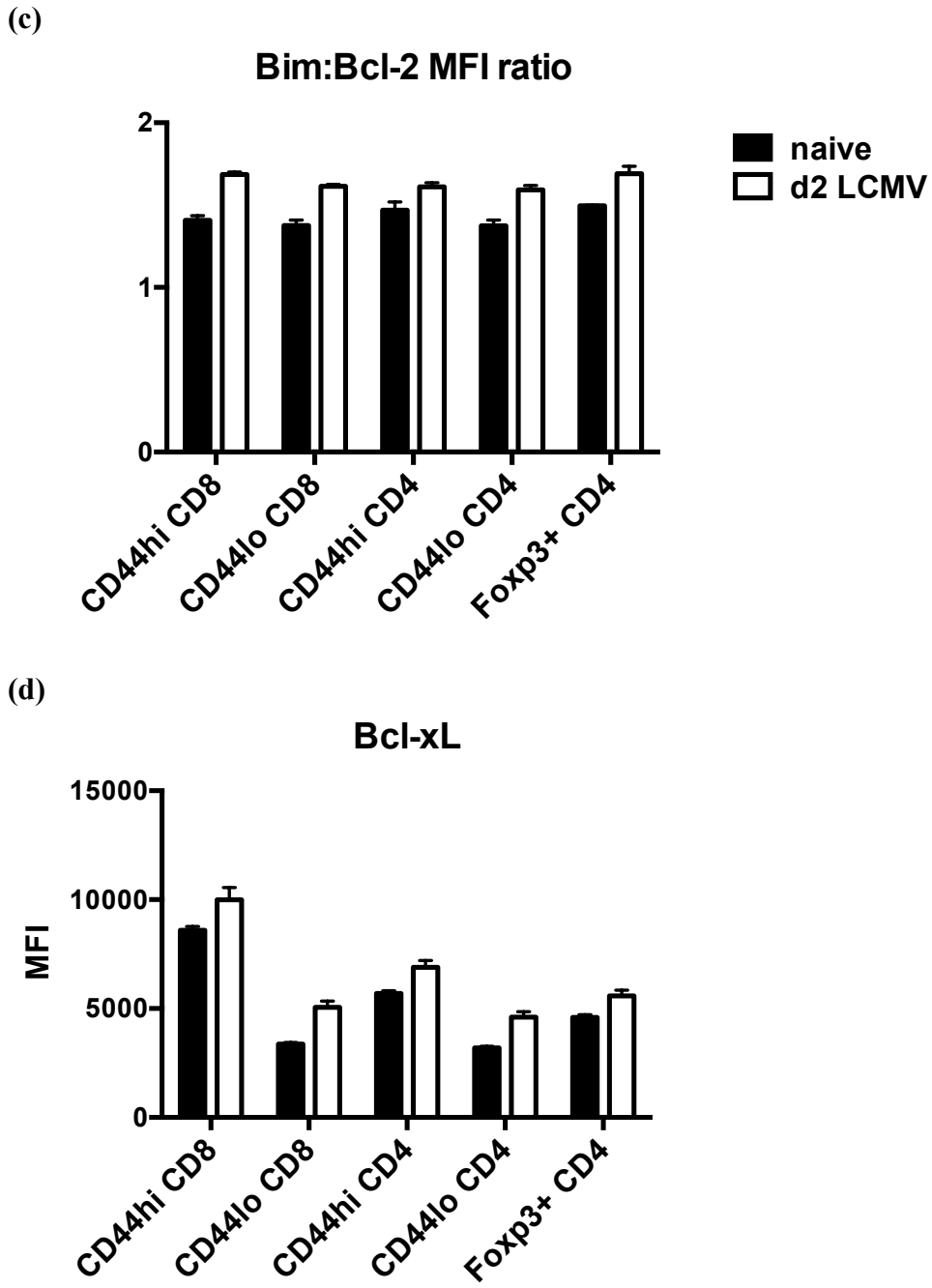


Figure 5.7.

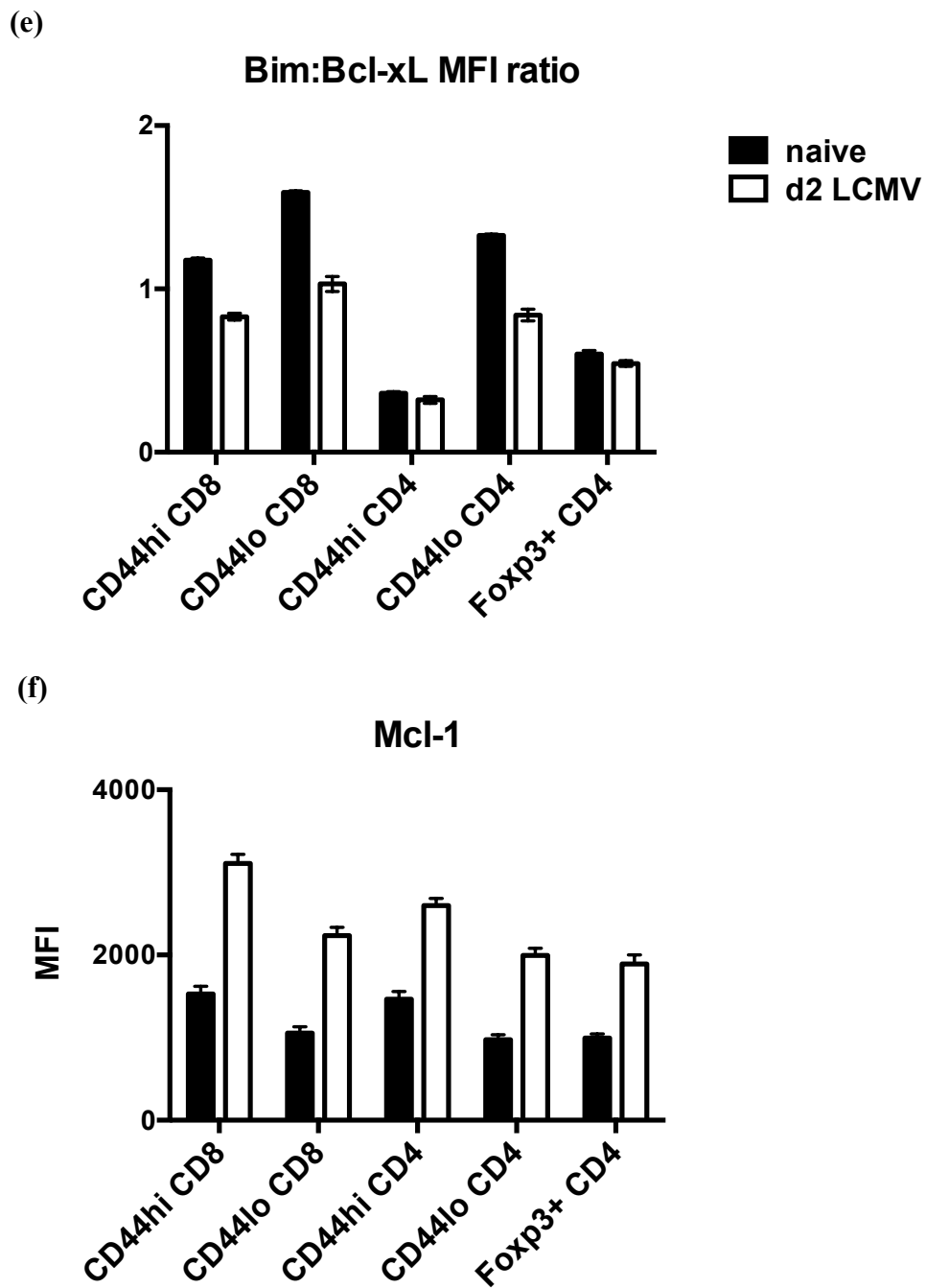
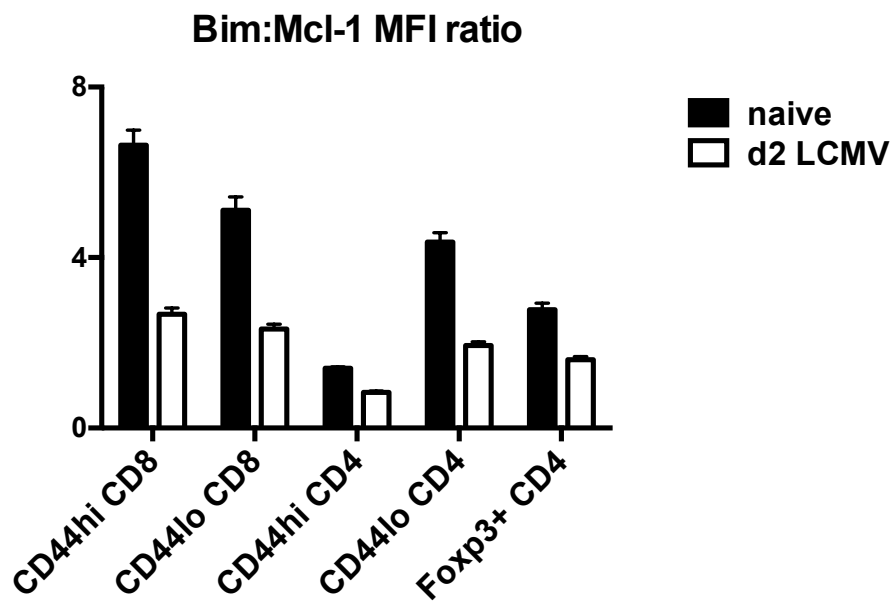


Figure 5.7.

(g)



(h)

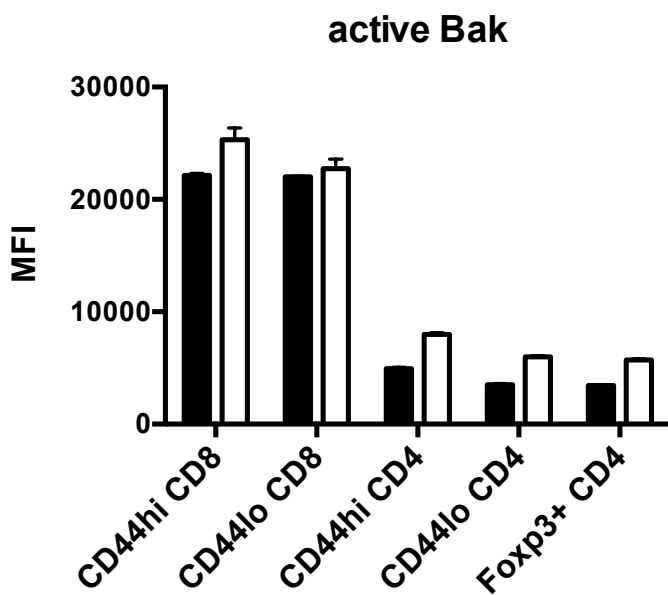
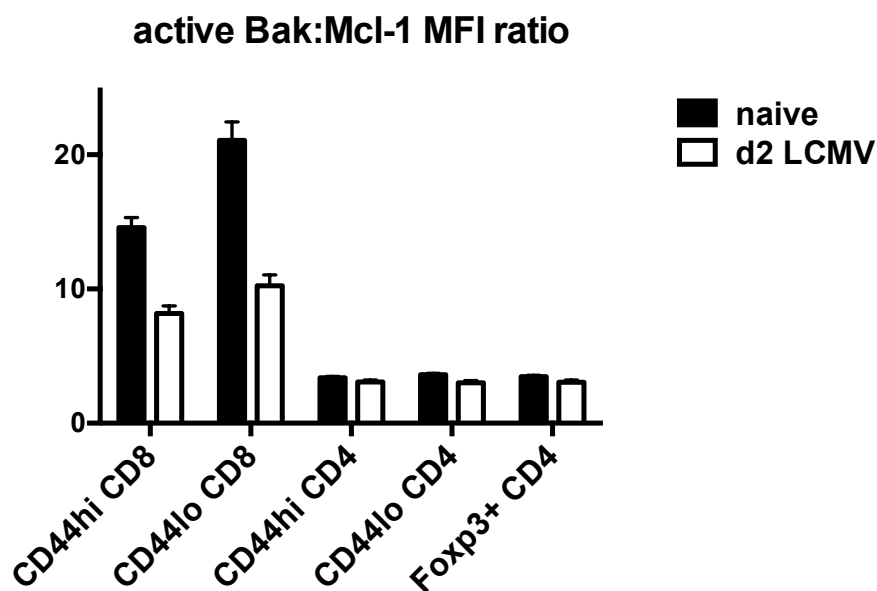


Figure 5.7.

(i)



(j)

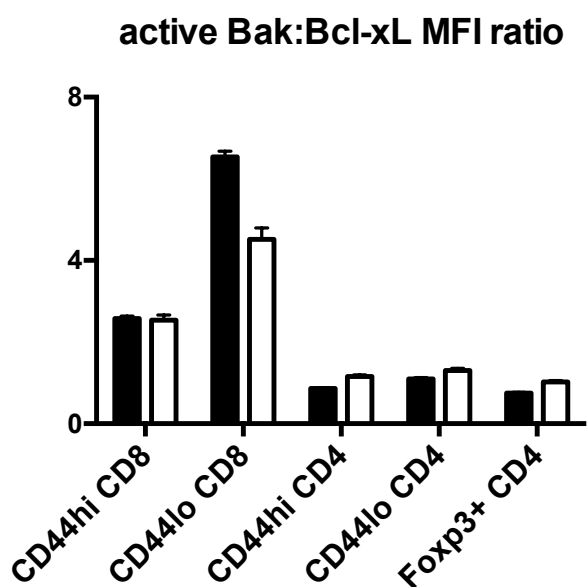


Figure 5.7. LCMV infection alters the balance between pro-apoptotic and anti-apoptotic molecules in T cells. Two days after i.p. infection with 5×10^4 pfu LCMV, splenocytes from C57BL/6J mice were stained *ex vivo* for Bcl-2 (a and c) and Bim (b, c, e and g); Bcl-xL (d, e and j); Mcl-1 (f, g and i); Bak NT (h-j). Geometric mean fluorescence intensity and the MFI ratio comparison are representative of at least two experiments (n=3).

Discussion

Previous work had established that CD44^{hi} CD8 and CD44^{hi} CD4 T cells are driven to apoptosis by virus- and by poly(I:C)-induced type I IFN (Bahl *et al.*, 2006; 2010; McNally *et al.*, 2001). The current study extends our knowledge to the Foxp3⁺ CD4⁺ natural Treg cells. Treg cells are major regulators of self-reactive T cells and are strong suppressors of autoimmunity. There are several experimental models of virus-induced autoimmunity in animals, and epidemiological correlates linking viral infections to the induction or exacerbations of a variety of autoimmune diseases, including diabetes, multiple sclerosis, rheumatoid arthritis, myocarditis, etc. (Kim *et al.*, 2006a; Van Der Werf *et al.*, 2007). A possible mechanism for virus-induced autoimmunity could be a consequence of the virus infection-induced apoptosis of Treg cells, because a momentary loss of Treg cells is enough to initiate an autoimmune disorder (Setoguchi *et al.*, 2005). However, most of the time viruses do not induce autoimmunity. It is not uncommon to see some self-reactive antibodies generated during infection (Berlin *et al.*, 2007; Sane *et al.*, 2012; Sjöwall *et al.*, 2012), but most of the time the host survives a viral infection without serious autoimmune manifestations. It is, therefore, of interest to determine whether Foxp3⁺ CD4⁺ natural Treg, present at the beginning of viral infections, are resistant or sensitive to the T cell apoptosis that occurs early during infection. Human Treg cells have been shown more resistant to apoptosis induced by irradiation or topoisomerase inhibitors than non-Treg CD4 T cells (Winzler *et al.*, 2011). Both murine and human Treg cells are resistant to activation-induced cell death (Banz *et al.*, 2002; Fritsching *et al.*, 2005). Here I showed that Treg cells were substantially more resistant

to virus infection-induced apoptosis than the CD44^{hi} CD4 and CD8 memory T cells with which they coexisted.

Treg cells might be more resistant to virus infection-induced apoptosis because of their ability to receive signals from IL-2. Both IL-7 and IL-2 have been shown to affect Treg population size in the periphery through regulating Foxp3 expression (Kim *et al.*, 2012; Zorn *et al.*, 2006). Foxp3 as a transcription factor can down-regulate the expression of CD127 but up-regulate CD25 expression (Williams & Rudensky, 2007), resulting in mature Treg cells being CD127^{lo} and CD25^{hi}, although recent work has also identified a CD127^{hi} Foxp3⁺ population in bone marrow and in skin (Simonetta *et al.*, 2010). Upon LCMV infection, CD127 expression was further reduced on Foxp3⁺ CD4⁺ Treg cells but the expression of CD25, CD122 and CD132 was either increased or unchanged. The ability to continue to receive survival signals through the IL-2/STAT5 pathway may allow Treg cells to survive and maintain functionality during infection. Attempts to block IL-2 signaling *in vitro* failed to consistently induce more apoptotic deaths in Treg cells during the 5-hr incubation prior to TUNEL assays. However, high dose (1mg/mouse) IL-2-neutralizing antibody has been shown to deplete Foxp3⁺ CD25⁺ CD4⁺ Treg cells over 3 days *in vivo* (Setoguchi *et al.*, 2005). The presence of IL-2 (0.1-10 ng/ml) has been shown to protect CD25⁺CD4⁺ Treg cells but not CD25⁻ conventional CD4 T cells from spontaneous and dexamethasone-induced cell death in a 12-hr *in vitro* assay (Chen *et al.*, 2004).

Could the sensitivity to apoptosis be the consequence of changes in cytokine receptors on T cells? IL-7R α (CD127) is thought to be down-regulated in response to T

cell activation. However, CD127 expression on WT donor CD8 T cells is much higher in IFNAR KO hosts than in WT hosts after acute LCMV infection (Nakayama *et al.*, 2010). Thus type I IFN probably indirectly down-regulates CD127 on T cells. While the expression of CD127 is important for naïve and memory CD8 T cell survival, signaling through the IL-7 receptor is not required during the initial proliferative response to an infection or an immunization (Schluns *et al.*, 2000). Decreasing the sensitivity of T cells to IL-7 through down-regulation of CD127 may be a way to allow selection and programming of responding T cells by other cytokines to generate specific responses to newly encountered antigen while simultaneously eliminating some pre-existing memory T cells.

LCMV infections change the ratio of pro- and anti-apoptotic molecules but no correlation to apoptosis induction is deduced. Previous attempts to decipher the mechanism of memory CD44hi CD8 T cell apoptosis and early attrition induced by type I IFN had not established any connection to NK cells, IFN γ R, perforin, FasL, granzyme B, TRAIL, TNFR1 or TNFR2 (McNally *et al.*, 2001)(Kapil Bahl's thesis). However, Bim is instrumental to the process. In Bim KO mice, the frequency of CD44hi CD8 T cells is relatively stable after LCMV infection, and the CD44hi CD8 T cells are significantly less TUNEL positive at day 3 post LCMV infection (Bahl *et al.*, 2010). Bim can physically interact with Bcl-2, Bcl-xL and Mcl-1 to initiate apoptosis (Hübner *et al.*, 2008; Willis *et al.*, 2007). The current data indicate no increase of Bim after LCMV infection, but Bcl-xL and Mcl-1 were up-regulated at day 2 post infection (Fig. 5.7). Changes in cytokine signals in the immune environment might have caused the up-regulation of anti-apoptotic

molecules. IL-2, IL-7 and IL-15 signaling can up-regulate the expression of Bcl-2, Bcl-xL and Mcl-1 (Lenz *et al.*, 2004; Opferman *et al.*, 2003), and certain co-stimulatory engagements can also increase Bcl-xL expression within hours of incubation (Lee *et al.*, 2002; van Oosterwijk *et al.*, 2007). Some anti-apoptotic molecules play a more important role in certain cells in a particular situation. Naïve CD8 T cells have a greater dependence on Bcl-2 to antagonize Bim than naïve CD4 T cells do (Wojciechowski *et al.*, 2007). Bcl-xL can rescue T cells from Fas-mediated cell death (Boise *et al.*, 1995), although its presence does not prevent LM-induced apoptosis of CD8 T cells (Jiang *et al.*, 2003b). Mcl-1 is important for T cell development and maintenance (Opferman *et al.*, 2003), and its role in Treg cell survival has recently been demonstrated (Pierson *et al.*, 2013). Since Treg cells are more resistant to virus infection-induced early apoptosis, it was expected that the relative abundance of anti-apoptotic and pro-apoptotic molecules would be different between Treg cells and the CD44hi CD8 and CD4 T cells. However, the Bim to anti-apoptotic molecule MFI ratios did not increase with the increased occurrences of apoptosis observed in the CD44hi CD8 and CD4 T cells after LCMV infection. In fact, the increased Bcl-xL and Mcl-1 expression after LCMV infection is counter-intuitive to what is expected of the CD44hi CD8 and CD44hi CD4 T cells with increased sensitivity to apoptosis and decreased IL-7 receptor expression. Therefore, the Bim to anti-apoptotic molecule MFI ratios may not be the appropriate correlates to apoptosis in this context.

CD8 and CD4 T cells may take different paths towards death. Bak becomes active and exposes the N-terminus while awaiting the second signal to become committed

to apoptosis. The level of active Bak was found to be much higher in CD8 T cells than in CD4 T cells and Treg cells. A preliminary observation was made that dying CD8 T cells were mostly caspase-3 and TUNEL double positive at day 3 post LCMV infection (data not shown). These pieces of evidence suggest that CD8 T cells undergo mitochondria- and caspase-dependent apoptosis after infection. On the other hand, CD4 T cells might have gone through more diverse forms of death apart from caspase-dependent apoptosis, since a good portion of TUNEL-staining dying cells were not caspase-3 positive (data not shown), and there were much lower levels of active Bak in CD4 T cells.

The spontaneous incidents of autoimmunity developed among patients receiving IFN α therapy for cancer (Rönblom *et al.*, 1991) and hepatitis (Dumoulin *et al.*, 1999; Fattovich *et al.*, 1996) and the association between infection and autoimmunity had us question whether virus-induced type I IFN might drive Treg into apoptosis and reduction in number, thereby allowing activation of autoreactive cells. This study indicates that the Foxp3⁺ CD4⁺ natural Treg population is relatively stable early during LCMV infection. A preliminary experiment with mice treated with a type I IFN inducer poly(I:C) for 1 day also showed more stable Foxp3⁺ Treg cells by TUNEL assay (data not shown), suggesting that type I IFN may not compromise the survival of natural Treg cells early during infection. This may explain the relatively low incidence of autoimmunity among type I IFN users and infected patients. Many other factors are involved in the development of autoimmunity, and further investigation will be needed to dissect the specific process for each autoimmune disease.

Chapter VI. Heterologous Immunity between LCMV and MCMV

The history of infections can influence the response to subsequent heterologous infections and the disease outcome. This is referred to as heterologous immunity, and it can be either beneficial or detrimental. Immunoprotection in terms of more rapid viral control or survival can be accompanied with enhanced or altered immunopathology depending on the biology of the disease. For examples, a history of LCMV infection can reduce the viral titer early after VACV infection and protect mice from lethal VACV challenge (Chen *et al.*, 2001a; Selin *et al.*, 1998), while immunopathology in the manifestation of panniculitis (acute fatty necrosis) in the visceral fat can also be developed in some of the i.p. infected LCMV-immune mice (Nie *et al.*, 2010; Selin *et al.*, 1998). On the other hand, a history of influenza infection not only enhances viral loads in subsequent LCMV and MCMV infections, but also increases the severity of lung immunopathology (Chen *et al.*, 2003).

Heterologous immunity has not been studied over the course of persistent infections. MCMV causes persistent infection and memory inflation in mice. Previous work has shown that LCMV infection is protective against MCMV and can reduce MCMV viral titer in the liver by about half a log pfu/ml early during infection (Selin *et al.*, 1998). This may potentially affect the generation of T cell responses and the establishment of persistent MCMV infection and viral latency. Sporadic reactivation of viral genome in infected cells is thought to be the source of antigen that leads to memory inflation, which describes the gradual accumulation of some MCMV-specific memory CD8 T cells beyond the initial expansion and contraction of responding T cell

populations (Karrer *et al.*, 2003; Seckert *et al.*, 2012). Changes in the viral control may potentially affect the process of memory inflation. On the other hand, the accumulated MCMV-specific memory CD8 T cells over time may interfere with responses to subsequent infections. T cell cross-reactivity between LCMV and MCMV will also be examined in this study using regular immune and long-term immune mice.

Results

More severe immunopathology in LCMV-immune mice

To study the long-term effect of heterologous immunity in MCMV infection, LCMV-immune mice and the sham control mice that were given only the supernatant from uninfected BHK cell cultures were infected with 5×10^5 PFU MCMV, and then examined at day 4, 12, 21, 42, 84 post infection. The first thing I noticed was the dramatic difference in immunopathology. When mice were inoculated intraperitoneally, the proximal fat in the lower abdomen became infected and inflamed. The necrosis in the fat pad was measured on a scale of 0-7 as published (Selin *et al.*, 1998). I found that on day 4 post MCMV infection, LCMV-immune mice showed severe necrosis in the fat pad while the sham control mice showed no sign of immunopathology (Fig. 6.1). Similar observations were made in subsequent repeat experiments. Necrosis was severe in both LCMV-immune and sham control groups at day 12 after the peak of T cell response. By day 21 most of the necrosis was resolved in the LCMV-immune mice, but prolonged necrosis was observed in some of the BHK control animals. I conclude that the history of LCMV infection causes more severe immunopathology early during MCMV infection.

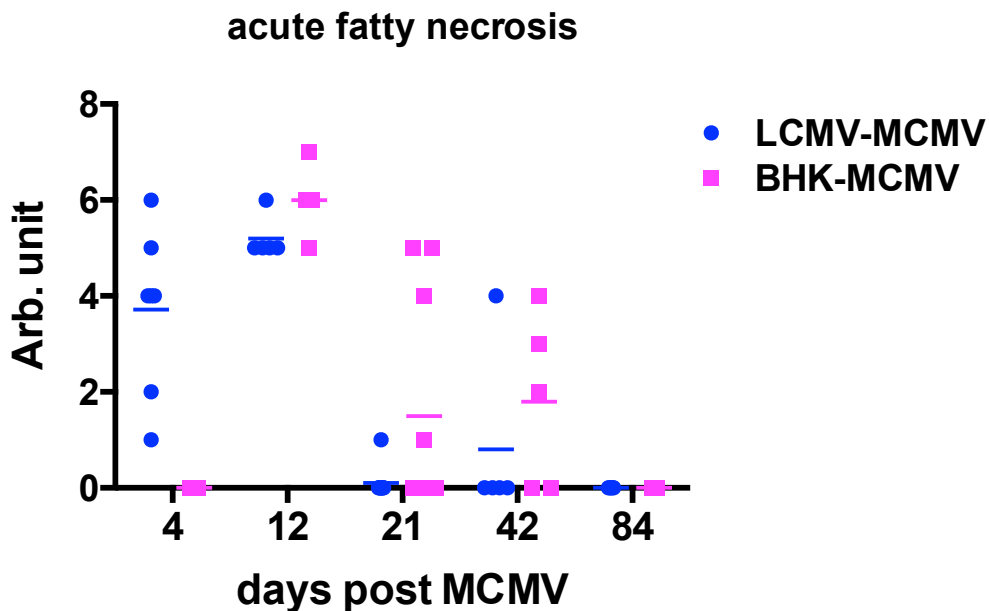


Figure 6.1. MCMV infection results in more severe acute fatty necrosis in the fat pads of LCMV-immune mice early during infection. LCMV-immune C57BL/6 mice and BHK sham controls were infected with 5×10^5 pfu MCMV. The necrosis in the fat pad was measured on a scale of 0-7 as published (Selin *et al.*, 1998): 1-2 means mild disease with only a few spots on the lower abdominal fat pad; 3-4 means moderate disease with larger white patches extending out to the upper quadrant of the fat pad; 5-6 is more severe with infiltration of inflammatory cells throughout the entire fat pad; and 7 is assigned if the organs are sticking together.

Higher MCMV viral loads in LCMV-immune mice

To evaluate the effect on viral control, MCMV viral copy number in genomic DNA extracted from snap-frozen solid organs was measured by qPCR using MCMV glycoprotein B (gB) primers for MCMV and mouse β -actin primers for the mouse genome. At day 4 post MCMV infection, more MCMV viral copies per μg of genomic DNA were found in the spleen, liver, kidney and lung of LCMV-immune mice than of the non-immune mice (Fig. 6.2a). A plaque assay also revealed a greater abundance of infectious MCMV particles in the liver of LCMV-immune mice in one of the experiments (data not shown). As expected, MCMV was not detected in the salivary gland for the first few days, and the viral copy numbers in both groups peaked on day 21 post MCMV infection (Fig. 6.2b). Viral growth kinetics were not affected by the history of LCMV infection; however, the viral load in the salivary glands of the LCMV-immune mice was higher than that of the BHK sham control mice throughout the entire experiment (Fig. 6.2b). Therefore, the history of LCMV infection enhanced viral burden during MCMV infection.

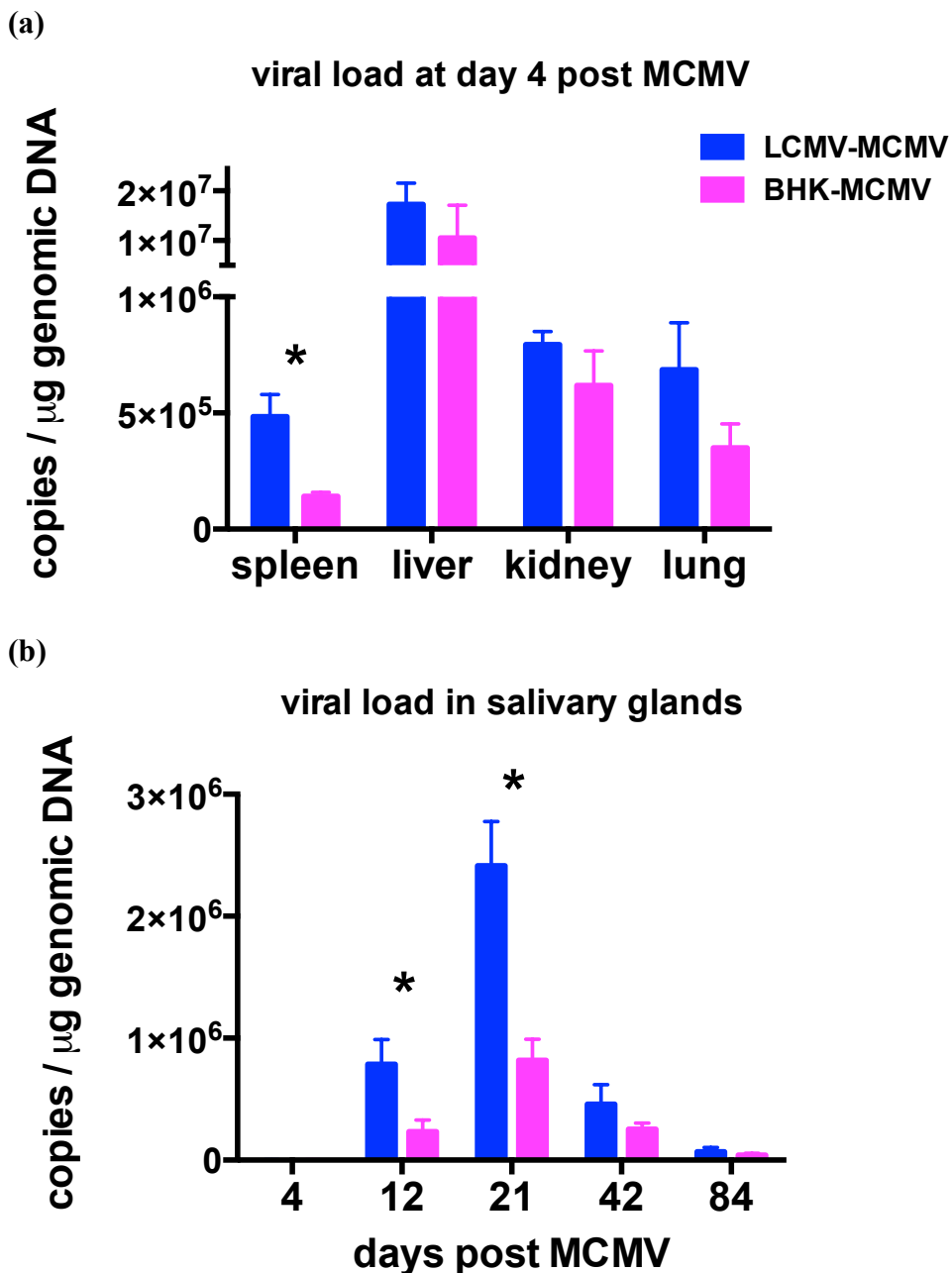


Figure 6.2. Viral loads are higher in mice with a history of LCMV infection. LCMV-immune C57BL/6 mice and BHK sham controls were infected i.p. with 5×10^5 pfu MCMV. MCMV copy number was measured by qPCR on DNA extracted from snap frozen organs. (a) MCMV viral load in the spleen, liver, kidney and lung on day 4 post infection. (b) MCMV viral load in the salivary glands on day 4, 12, and week 3, 6, 12 post infection. (*) denotes statistical significance ($p < 0.05$) between LCMV-immune and BHK control groups in the specific organ or at the specific time-point ($n=5$).

Fewer MCMV-specific inflationary memory CD8 T cells in LCMV-immune mice

To examine the effect on T cell response to MCMV infection and T cell memory generation, MCMV-specific CD8 T cells in the spleen were analyzed by ICS after *in vitro* peptide stimulation. After the period of memory inflation at week 12 post MCMV infection, the numbers of inflationary memory CD8 T cells specific for m139₄₁₉₋₄₂₆, M38₃₁₆₋₃₂₃ and IE3₄₁₆₋₄₂₃ were lower in the LCMV-immune mice than in non-immune controls (Fig. 6.3a). The number of M38₃₁₆₋₃₂₃-specific memory CD8 T cells is the most consistently accrued to a higher number in BHK control mice across repeat experiments. No difference was found in the number of non-inflationary memory T cells between the LCMV-immune and the sham control groups at 12 weeks post infection (Fig. 6.3b). The suppressed memory inflation was also evident in the number of CD8 T cells accumulated in the spleen over time. The total CD8 T cell number was significantly higher in the BHK control group than in LCMV-immune group at 12 weeks post MCMV infection (Fig. 6.3c). Memory CD44^{hi} CD8 T cells were also accrued to a higher number in the BHK control group than the LCMV-immune group at week 6 and 12 post infection (Fig. 6.3d). Therefore, the history of LCMV infection reduced the accumulation of inflationary memory CD8 T cells in the spleen.

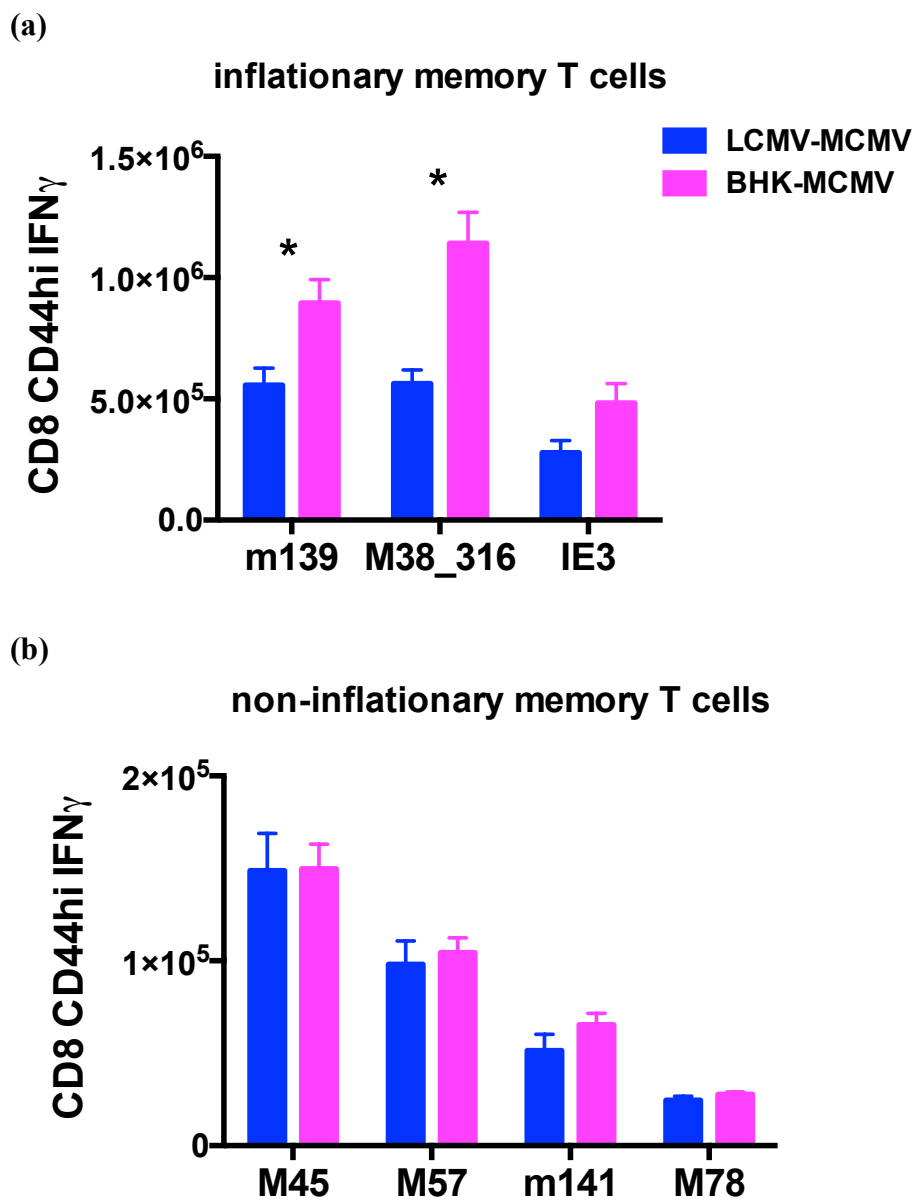


Figure 6.3.

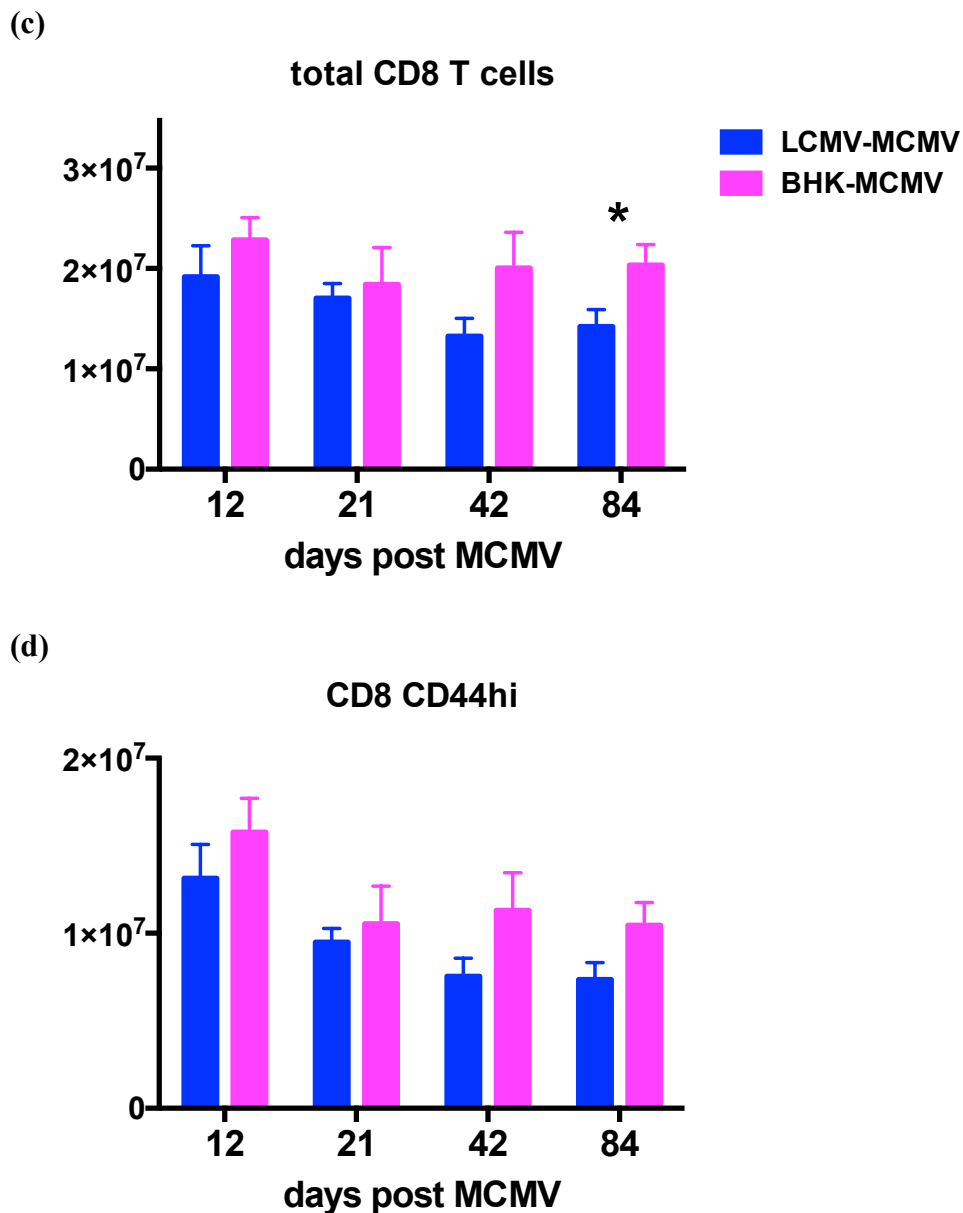


Figure 6.3. A history of LCMV infection suppresses the generation of inflationary memory T cells. LCMV-immune C57BL/6 mice and BHK sham controls were infected i.p. with 5×10^5 pfu MCMV. Splenocytes harvested at day 12, 21, 42 and 84 post MCMV infection were assayed by ICS. LCMV-immune mice accumulated less inflationary memory T cells (a) at week 12 (day 84) post infection but the numbers of non-inflationary memory T cells (b) were comparable between the LCMV-immune and BHK control groups. Total CD8 T cells (c) and memory CD44hi CD8 T cells (d) were accrued to a higher number in the non-immune control group. (*) denotes statistical significance ($p < 0.05$) between LCMV-immune and BHK control groups with the specific epitopes or at the specific time-point (n=5).

Stronger NP₃₉₆₋₄₀₄-specific CD8 T cell response in MCMV-immune mice

T cell cross-reactivity may be one of the ways heterologous immunity affects immune responses to subsequent infections with unrelated pathogens. To examine whether cross-reactive epitopes exist between LCMV and MCMV, LCMV-immune mice and the BHK controls were examined at day 8 post MCMV infection by cytokine staining after *in vitro* stimulation of splenocytes with 20 different peptides separately (Appendix Table 1) (Munks *et al.*, 2006b). Despite the variability between experiments, no substantial and repeatable cross-reactive response was identified with the sequence of infections. The history of LCMV infection had no dramatic effect on the magnitude or the hierarchy of CD8 T cell response to MCMV on day 8 post infection (Fig. 6.4). The higher viral burden among LCMV-immune mice did not increase the magnitude of CD8 T cell response to MCMV.

The reciprocal experiment was done with MCMV-immune and nsg sham control mice. Eight days after infection with 5×10^4 pfu LCMV, splenocytes were stimulated with 16 different peptides separately (Appendix Table 2) (Kotturi *et al.*, 2007). While the age-matched nsg sham control mice mounted a variable response to LCMV with a dominant GP₃₃₋₄₁ response in one out three mice and a dominant NP₃₉₆₋₄₀₄ response in another, LCMV infection induced a robust response of a higher overall magnitude in all three MCMV-immune mice (Fig. 6.5a). The response to NP₃₉₆₋₄₀₄, L₆₈₉₋₆₉₇ and L₃₃₈₋₃₄₆ was significantly greater in the MCMV-immune mice than in the nsg controls (Fig. 6.5b). Changes in the magnitude of response to some peptides in the immune mice may suggest the existence of low-affinity cross-reactivity, and further investigation will be needed.

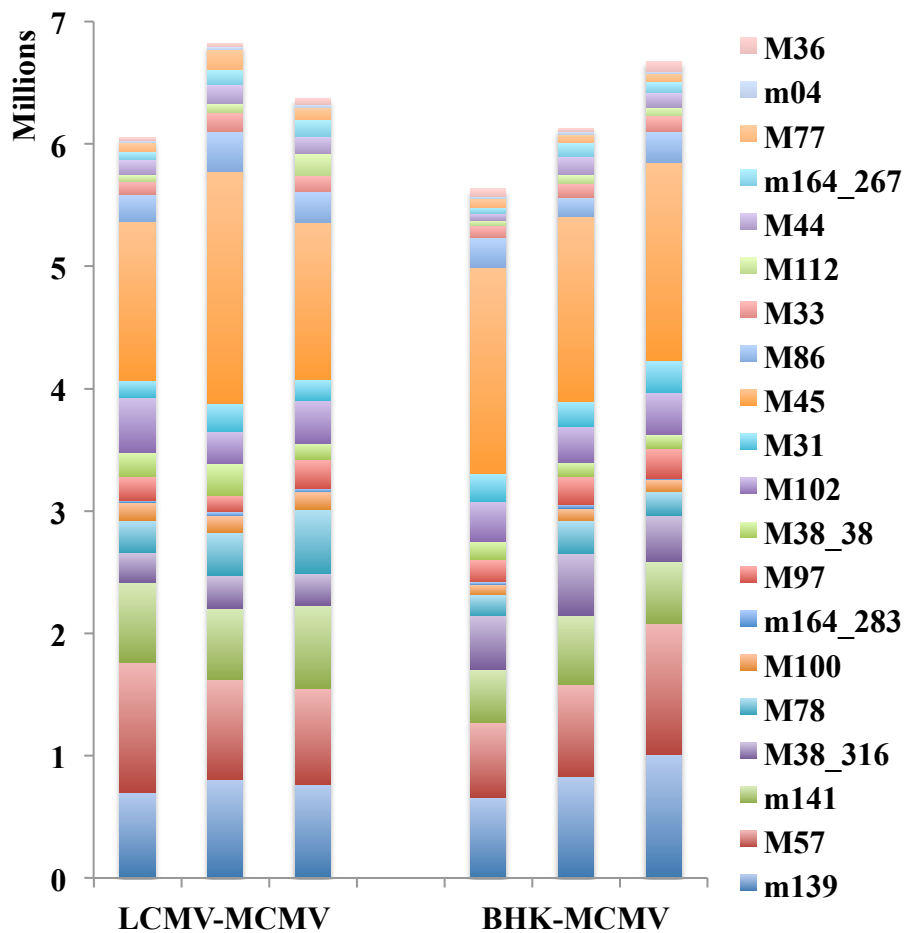


Figure 6.4. LCMV-immune mice display no apparent cross-reactive CD8 T cells response to MCMV during acute infection. LCMV-immune mice and BHK sham controls were infected i.p. with 5×10^5 pfu MCMV. Splenocytes were analyzed by ICS at day 8 post infection.

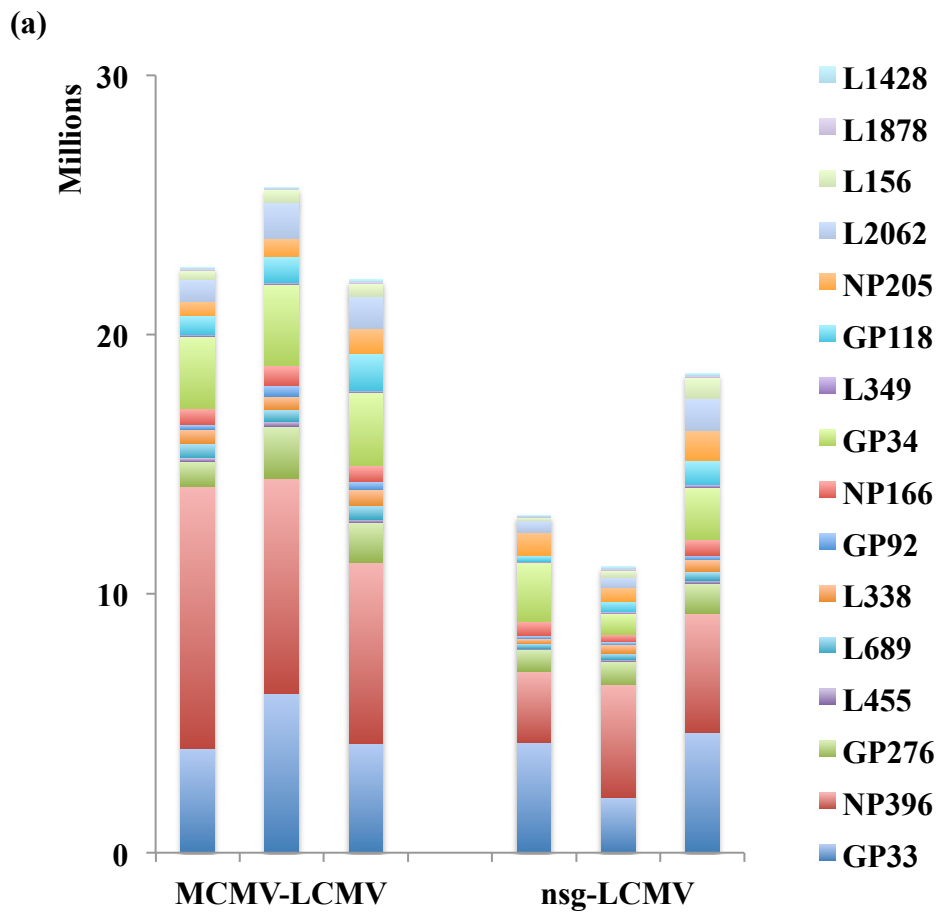


Figure 6.5.

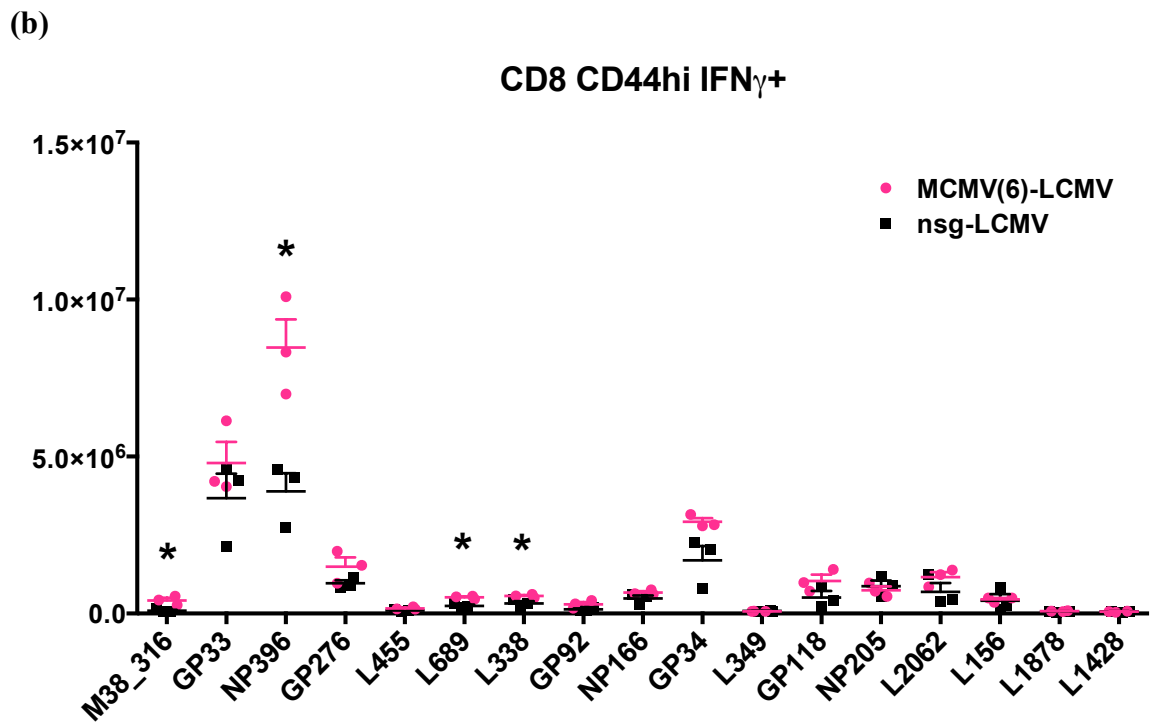


Figure 6.5. LCMV infection induces a stronger NP₃₉₆₋₄₀₄-specific response in MCMV-immune mice. MCMV-immune mice and nsg sham controls were infected with 5×10^4 pfu LCMV. Splenocytes were analyzed by ICS at day 8 post infection. (a) MCMV-immune mice displayed a robust CD8 T cell response to LCMV. (b) NP₃₉₆₋₄₀₄-specific CD8 T cell response was significantly stronger in the MCMV-immune mice than in nsg sham controls. (*) denotes statistical significance ($p < 0.05$) between MCMV-immune and nsg control groups with the specific epitopes.

Decline in T cell response in aging mice unrelated to history of MCMV infection

The association between human cytomegalovirus and the aging of the immune system (a.k.a. immune senescence) has been suggested (Moss, 2010; Pawelec *et al.*, 2009; 2010). One of the signs of immune senescence is the decreased ability to respond to new antigens (Goronzy & Weyand, 2013). To examine whether a history of MCMV infection, independent of immune senescence, may affect the immune response to a subsequent heterologous infection, mice inoculated with MCMV or nsg sham controls were subsequently infected with 5×10^4 pfu LCMV, a non-lethal dose in adult mice, at weeks 30, 45, 60, 75, 90, 91, 105, 120 and 130 post MCMV infection. As expected, the magnitude of the peptide-specific T cell response to LCMV at day 8 post LCMV infection weakened gradually with age, especially after week 60 post MCMV infection, but the response was not dramatically different between the long-term MCMV-immune mice and the nsg sham controls (Fig. 6.6). Over time the percentage of response to CD8 peptides GP₃₃₋₄₁, GP₂₇₆₋₂₈₆ and NP₃₉₆₋₄₀₄ did not decline as much as the percentage of response to CD4 peptide GP₆₁₋₈₀ did (Fig. 6.7). Either a hampered proliferative response or a decreased number of precursor CD8 T cells with age might have led to the reduction of responding CD8 T cell number. Although a decline in the T cell response was observed with age, the long-term MCMV infection did not appear to cause more impairment of the T cell response to LCMV infection.

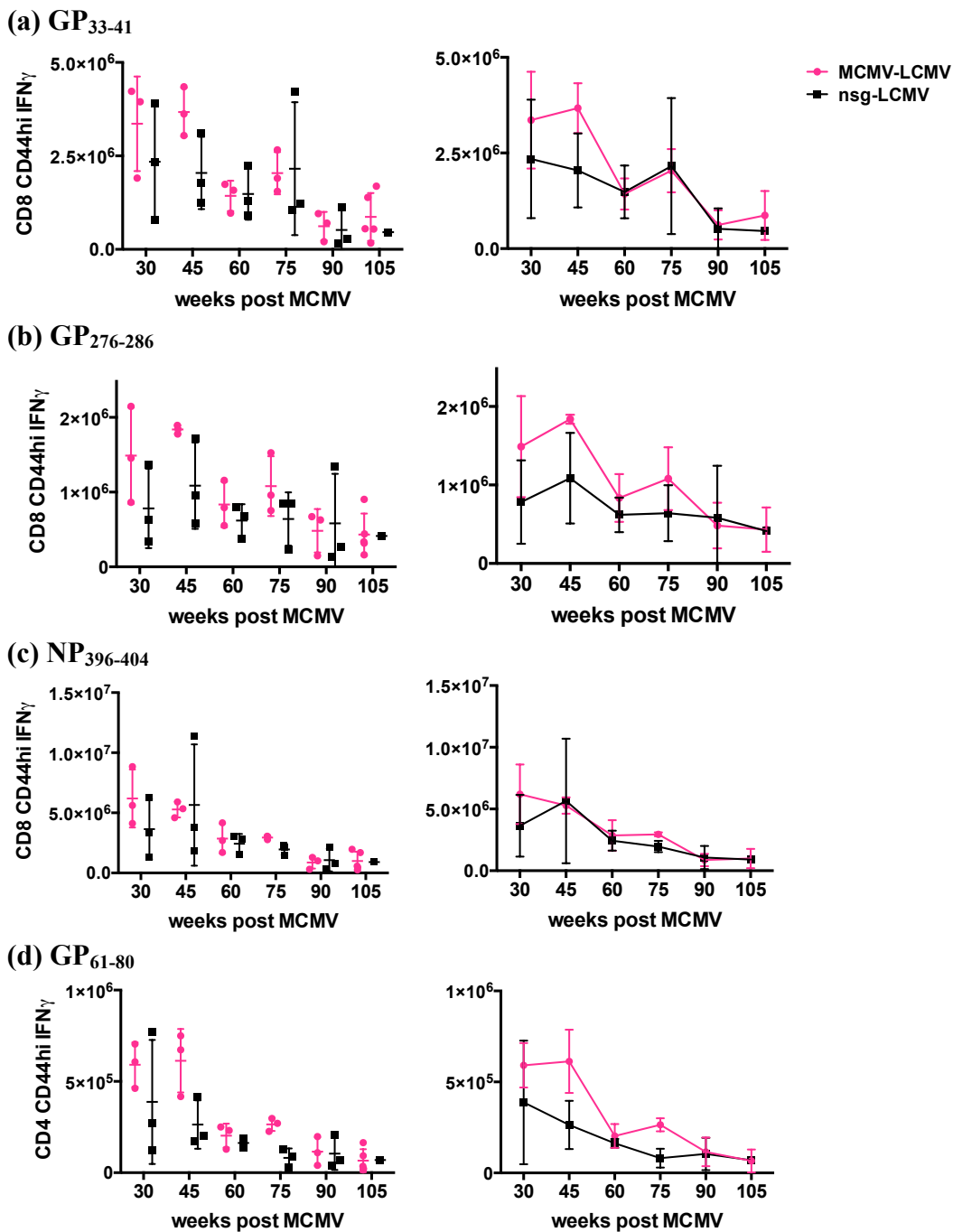


Figure 6.6. The magnitude of T cell response to LCMV infection declines with age. MCMV-immune and nsg control mice infected with 5×10^4 pfu LCMV at different time-points post MCMV infection were analyzed at day 8 by ICS. CD8 T cell responses to GP_{33-41} (a), $GP_{276-286}$ (b) and $NP_{396-404}$ (c) and CD4 T cell response to GP_{61-80} (d) are shown by individuals in group plots on the left and as line graphs with mean and SEM on the right.

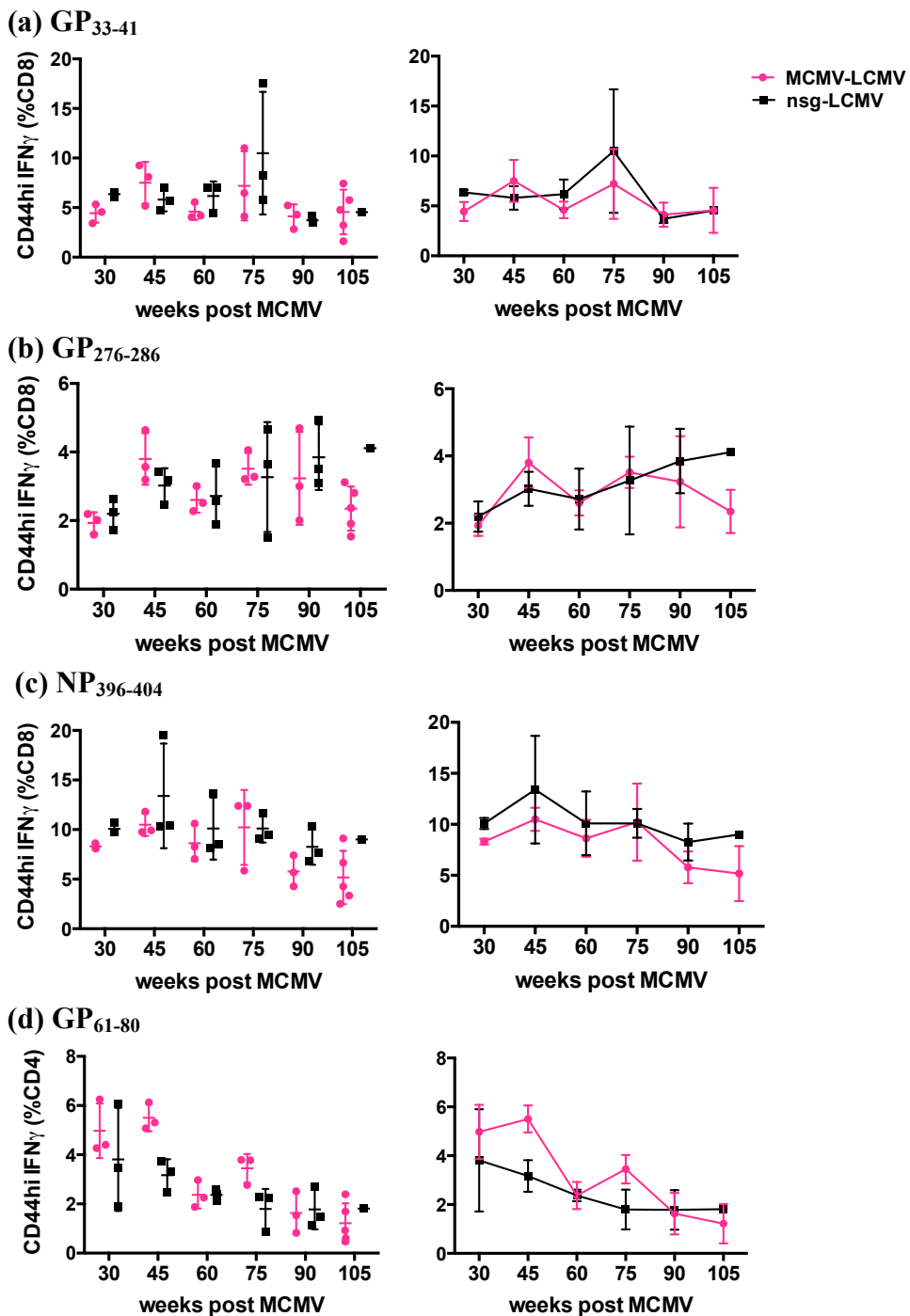


Figure 6.7. The percentage of response to CD4 epitope GP₆₁₋₈₀ declines with age, but the percentages of response to CD8 epitopes are more stable over time. T cell responses of MCMV-immune and nsg control mice to LCMV were analyzed at day 8 post infection by ICS. The percentage of responses by individuals in group plots are shown on the left and as line graphs with mean and SEM on the right.

Novel cross-reactive MCMV peptide M57₇₂₇₋₇₃₄ identified

When MCMV-long-term-immune mice were infected with LCMV, several mice displayed an elevated response to LCMV peptide L₂₀₆₂₋₂₀₆₉: 15.7%, 21.4%, 6.9%, 12.4% of CD8 T cells were responsive to L₂₀₆₂₋₂₀₆₉, compared to normally only 1-2% responding (Fig. 6.8a). In approximately one in every five MCMV-immune mice, the hierarchy of the epitope response to LCMV was altered, and nearly 50% of the response became L₂₀₆₂₋₂₀₆₉-specific (Fig. 6.8b and 6.8c). L₂₀₆₂₋₂₀₆₉ is a K^b-restricted epitope. Using Basic Local Alignment Search Tool (BLAST®) search against the MCMV protein sequence, 14 peptide sequences with substantial homology to the original L₂₀₆₂₋₂₀₆₉ sequence and 28 sequences with homology to the its variations were identified. Two new peptides M57₇₂₇₋₇₃₄ and m165₁₁₄₋₁₂₁ with a SYPEITHI score of 19 and 20 respectively were synthesized and tested along with M100₇₂₋₇₉ (Fig. 6.9a) by ICS at day 8 post LCMV infection. MCMV-immune mice with an elevated response to L₂₀₆₂₋₂₀₆₉ responded to M57₇₂₇₋₇₃₄ with a similar magnitude and altered the proportional response hierarchy (Fig. 6.9b), suggesting that M57₇₂₇₋₇₃₄ is cross-reactive with L₂₀₆₂₋₂₀₆₉-specific CD8 T cells. In contrast, no response was raised against m165₁₁₄₋₁₂₁, suggesting that C57BL/6 mice might not recognize m165₁₁₄₋₁₂₁ as an epitope. To see if the cross-reactive epitopes were recognized by the same subset of TCR clones, V β usage of L₂₀₆₂₋₂₀₆₉- and M57₇₂₇₋₇₃₄-specific T cells was analyzed in one nsg control mouse and two MCMV-immune mice after infection with LCMV at week 120 post MCMV infection. While both M57₇₂₇₋₇₃₄- and L₂₀₆₂₋₂₀₆₉-specific T cells preferentially used V β 5.1/5.2 and V β 8.1/8.2, L₂₀₆₂₋₂₀₆₉-specific T cells could additionally use V β 13, V β 14 and V β 17a (Fig. 6.10).

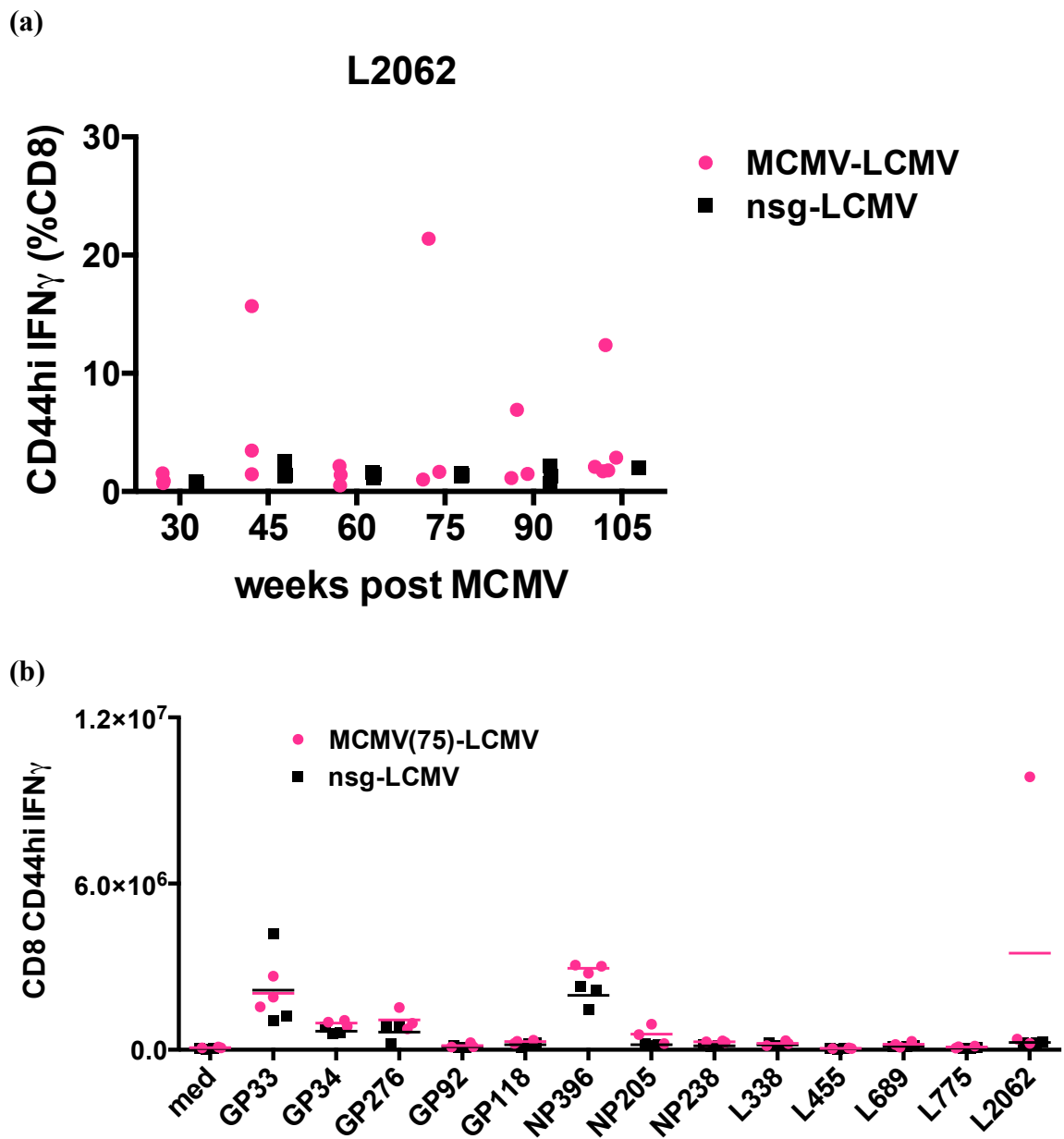


Figure 6.8.

(c)

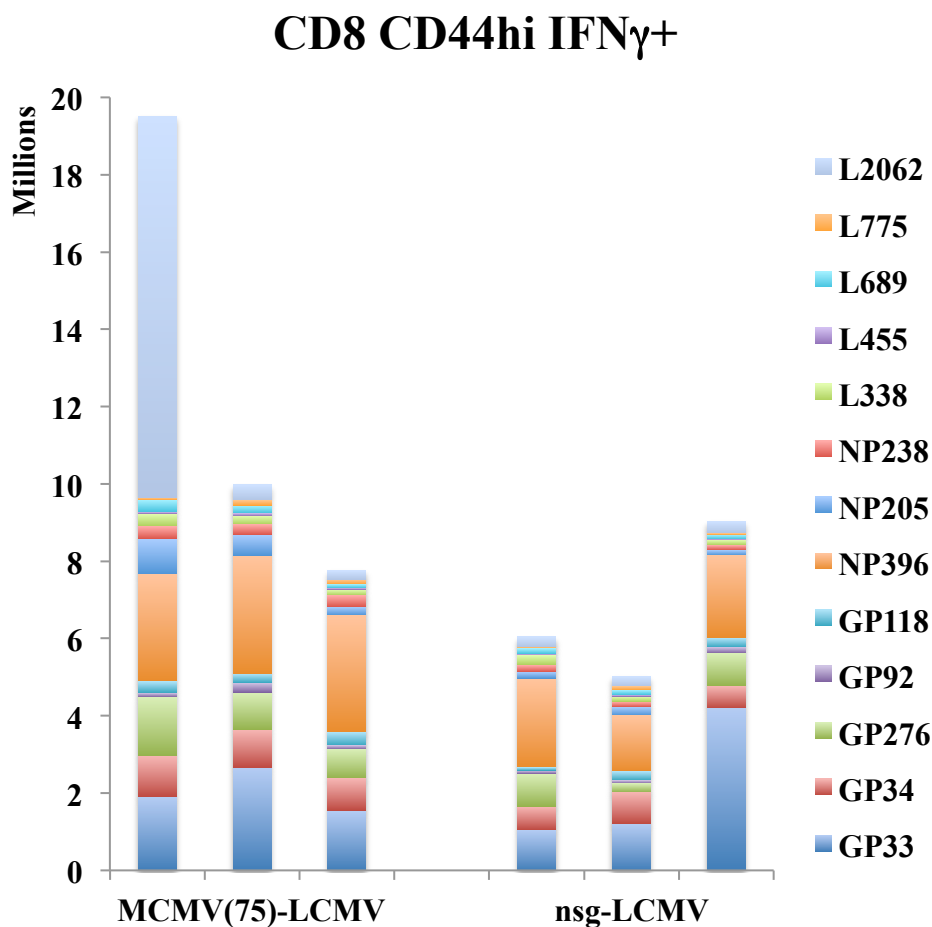


Figure 6.8. MCMV-long-term-immune mice show possible cross-reactive response to LCMV L₂₀₆₂₋₂₀₆₉. T cell responses of MCMV-immune and nsG control mice to LCMV were analyzed at day 8 post infection by ICS. (a) High frequency response to L₂₀₆₂₋₂₀₆₉ was found in several MCMV-immune mice at different time-points post MCMV infection. (b and c) Representative data at week 75 post MCMV infection show a possible cross-reactive response to L₂₀₆₂₋₂₀₆₉ and normal responses to other LCMV epitopes.

(a)

LCMV	L ₂₀₆₂₋₂₀₆₉	R	S	I	D	F	E	R	V
MCMV	M57 ₇₂₇₋₇₃₄	K	S	V	E	F	E	R	I
	m165 ₁₁₄₋₁₂₁	R	G	S	D	F	S	R	V
	M100 ₇₂₋₇₉	R	I	I	D	F	D	N	M

(b)

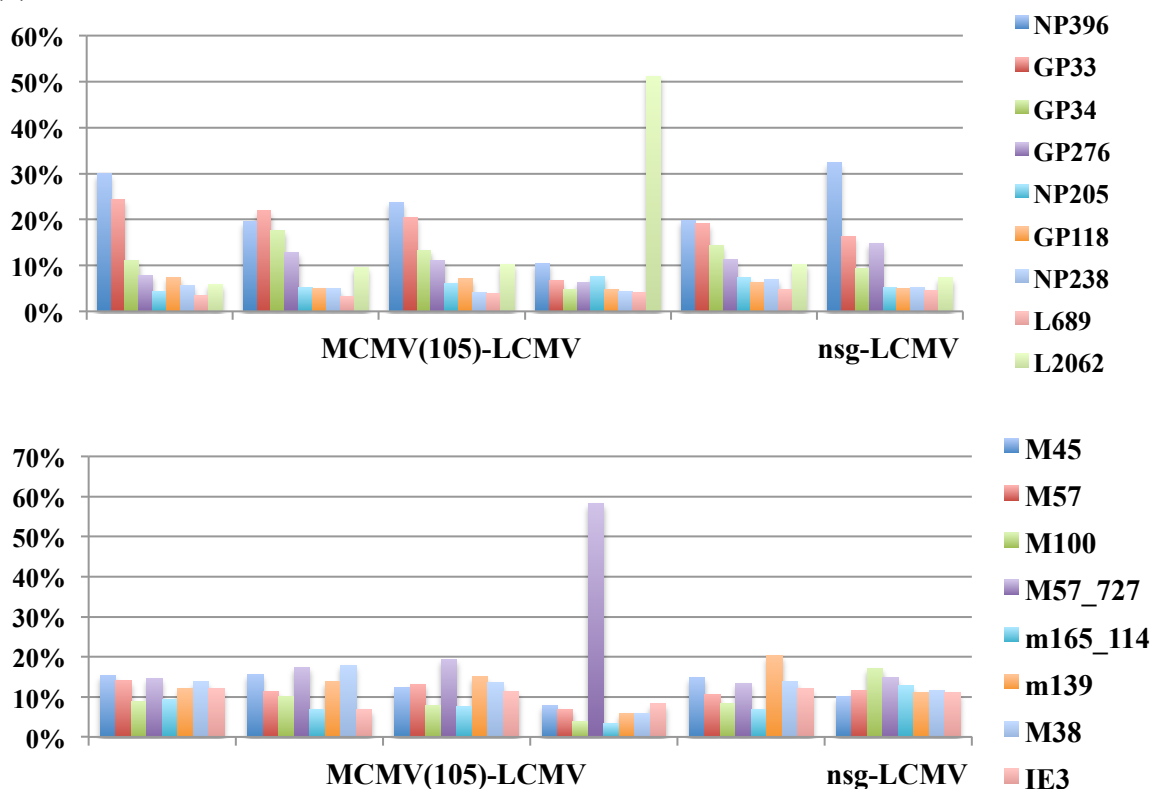


Figure 6.9. MCMV M57₇₂₇₋₇₃₄ is probably cross-reactive to LCMV L₂₀₆₂₋₂₀₆₉. (a)

Three MCMV peptides with sequence homology to L₂₀₆₂₋₂₀₆₉ were chosen for experiments. (b) Five MCMV-immune (week 105 post MCMV infection) mice and one nsg control mouse were analyzed at day 8 post LCMV infection. About 50% of major LCMV-specific response was found specific for L₂₀₆₂₋₂₀₆₉ in the same mouse that showed over 50% of major MCMV-specific response specific for M57₇₂₇₋₇₃₄.

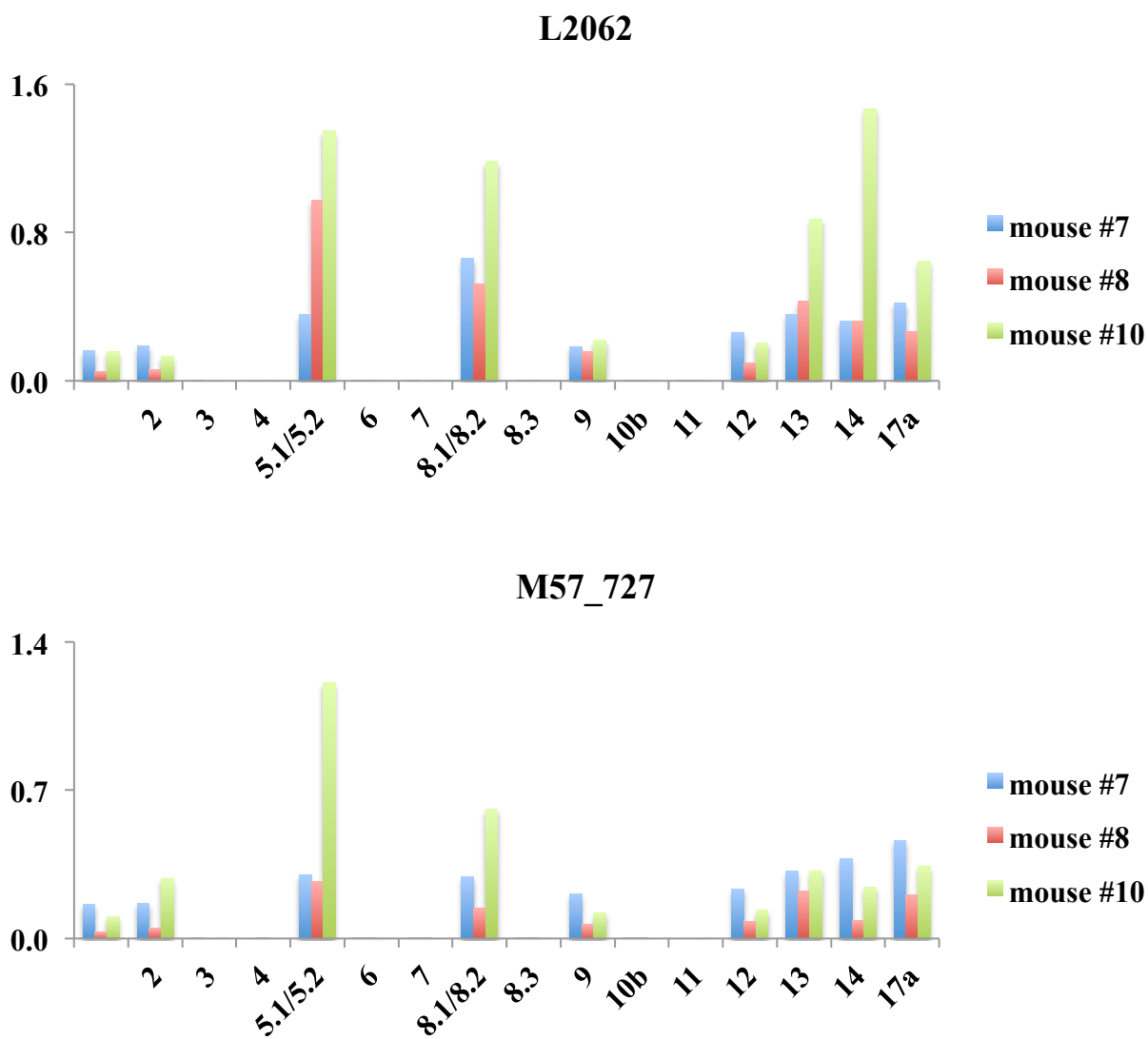


Figure 6.10. LCMV $L_{2062-2069}$ - and MCMV $M57_{727-734}$ -specific T cells both use $V\beta_{5.1/5.2}$ and $V\beta_{8.1/8.2}$. MCMV-immune (week 120 post MCMV infection; mouse #8 and #10) and nsg control (mouse #7) mice were analyzed at day 9 post LCMV infection by ICS. The frequencies of $IFN\gamma^+ V\beta^+$ (% CD8 CD44^{hi}) T cells were compared by different V β in each mouse.

Discussion

The history of exposure to pathogens can influence the host response to subsequent infections and the disease outcome. MCMV causes persistent infection and CD8 memory T cell inflation in mice. This study examined the effect of heterologous immunity in persistent infection using LCMV and MCMV infections, and found that the history of LCMV infection caused more severe panniculitis early during MCMV infection. The more severe panniculitis may not be a surprise in LCMV-immune mice, since similar immunopathology has been observed after VACV infection. Preliminary experiments showed that, after MCMV infection, macrophages infiltrated into the visceral fat pads, and the CD8 T cells and NK1.1+ cells were much more abundant in the fat pads of LCMV-immune mice than non-immune controls at day 4 post MCMV infection. The cellular infiltrates along with the TNF produced by macrophages could accelerate the necrosis of fat cells, causing visible pathology.

Higher MCMV viral loads were observed in the spleen, liver, kidney, lung and salivary glands in the LCMV-immune mice. The qPCR is a very sensitive tool for viral genome detection, although this technique does not differentiate between infectious and non-infectious particles. A previous study examining heterologous immunoprotection by plaque assay showed that immunity to LCMV was protective against MCMV, and reduced the MCMV titer by about half a log in comparison to naïve mice challenged with MCMV (Selin *et al.*, 1998). In this study, although the MCMV copy numbers were consistently higher in the LCMV-immune mice, viral titers by plaque assays sometimes showed no difference between LCMV-immune and BHK sham control mice in repeat

experiments. Plaque assay may not be as robust as qPCR because slight changes in infectivity or errors in serial dilutions may deviate the plaque counts.

Higher MCMV viral titers early during infection were expected to enhance memory inflation later on because more cells would be seeded with latent MCMV and thereby a greater chance of stimulating memory CD8 T cell inflation by viral recurrence. However, the number of inflationary memory CD8 T cells was found reduced in the LCMV-immune mice. The percentage of lymphocytes that were CD8 positive was steadily maintained in both groups at about 20% throughout the study, but the number of CD8 T cells and the number of CD44^{hi} CD8 T cells were lower in the LCMV-immune group. This observation may suggest that instead of the limited space or cytokine resources, some other mechanism(s) may be involved in suppressing the generation of inflationary memory in the LCMV-immune mice. Memory inflation depends on peptide processing by constitutive proteasomes and presentation by non-hematopoietic cells (Hutchinson *et al.*, 2011; Seckert *et al.*, 2011; Torti *et al.*, 2011a). It may be possible that these cells are affected by the history of LCMV infection and become less active in stimulating the inflationary memory T cells. Non-hematopoietic cells have been shown to participate in stimulating proliferation of LCMV-specific CD8 T cells (Thomas *et al.*, 2007). LCMV and MCMV might have infected different types of non-hematopoietic cells that are in the proximity of each other in the same secondary lymphoid organs (Mueller & Germain, 2009), and may possibly affect latency establishment and viral reactivation of the virus that comes later in the infection sequence.

MCMV did not contribute to immune senescence in mice. Human cytomegalovirus has been suggested to associate with immune senescence (Karrer *et al.*, 2009; Moss, 2010; Pawelec *et al.*, 2010), but the hypothesis has not been directly tested. In this study, the long-term MCMV-immune mice generated comparable CD8 and CD4 T cell responses to LCMV as the age-matched sham control mice did. The decline in T cell responses to LCMV over time in both groups is consistent with the phenomenon of immune senescence (Agarwal & Busse, 2010). A recent study comparing young and aged mice showed that aged mice previously inoculated with the MCMV deletion mutant MCMV- Δ 157, which lacks the ligand m157 for NK cell activating receptor Ly49H, are impaired in the GP₃₃₋₄₁- and NP₃₉₆₋₄₀₄-specific CD8 T cell response to LCMV infection, resulting in a higher LCMV viral load than the aged-matched controls (Mekker *et al.*, 2012). MCMV- Δ 157 is not as well controlled by NK cells as the parent strain in C57BL/6 mice, resulting in a higher initial viral burden (Bubić *et al.*, 2004); however, viral persistence should have been resolved in the aged mice, and thus the different strains of MCMV used may not account for the different observations in the two studies. I did not compare LCMV viral titer after infection, but a MCMV-immune mouse that succumbed to LCMV infection was found unable to control infection even on day 11, which is unusual with the use of low dose LCMV Armstrong strain. Nevertheless, the overall mortality rate was similar between MCMV-immune and non-immune control groups whether the mice were subsequently infected with LCMV or not. Therefore, MCMV did not seem to intensify the effect of immune senescence in aged mice in this study.

In the long-term MCMV-immune mice infected with LCMV, a new MCMV epitope sequence M57₇₂₇₋₇₃₄ was found to possibly cross-react with LCMV L₂₀₆₂₋₂₀₆₉. These two sequences are the first cross-reactive epitope pair identified between LCMV and MCMV. M57₇₂₇₋₇₃₄ shares 50% sequence homology with L₂₀₆₂₋₂₀₆₉. The rest of the amino acids are also biochemically similar, with a bulky basic residue (Lys vs Arg) in position 1, hydrophobic residues (Val vs Ile) in position 3 and 8, and an acidic residue (Glu vs Asp) in position 4. Therefore, MHC-M57₇₂₇₋₇₃₄ complex may potentially interact with the same TCR that recognize MHC-L₂₀₆₂₋₂₀₆₉ through molecular mimicry (Hagerty & Allen, 1995; Welsh & Selin, 2002). In this study, cross-reactivity was observed approximately once in every five MCMV-immune mice, consistent with the phenomenon of private specificity, where only some T cell repertoires may respond to the cross-reactive epitope of the other virus. Experiments suggested that M57₇₂₇₋₇₃₄ is a subdominant epitope, but when stimulated in the context of LCMV infection, the M57₇₂₇₋₇₃₄-specific T cells greatly increased in number and dominated the virus-specific memory T cell pool. At the same time, the response to subdominant LCMV epitope L₂₀₆₂₋₂₀₆₉ became the major CD8 T cell response. This alteration of immunodominance hierarchy is very similar to NP₂₀₅₋₂₁₂ in LCMV and PV infections, where a prior history of infection with one virus will greatly alter the hierarchy of the CD8 T cell response to the other virus (Brehm *et al.*, 2002).

Chapter VII. Non-reciprocal heterologous protection between LCMV and VACV

The protection conferred by heterologous immunity is not necessarily reciprocal. Immunity to pathogen A being protective against pathogen B does not guarantee that the immunity to pathogen B will protect against pathogen A. A history of infection with LCMV, MCMV, PV, BCG or influenza A virus provides a certain level of heterologous protective immunity against VACV in the form of reduced organ viral titer by one to two log pfu early (day 3-4) after infection (Chen *et al.*, 2003; Mathurin *et al.*, 2009; Selin *et al.*, 1998). However, a prior history of VACV infection does not protect against subsequent challenge with LCMV, MCMV or PV (Selin *et al.*, 1998).

Protection against VACV in LCMV-immune mice is mediated by both CD8 and CD4 T cells through IFN γ . IFN γ -producing CD8 and CD4 T cells are recruited in LCMV-immune mice to the site of VACV infection (Chen *et al.*, 2001a; Selin *et al.*, 1998). Anti-IFN γ -treated or IFN γ R KO LCMV-immune mice are no longer protected against VACV, and recipients of CD8 or CD4 T cell-depleted LCMV-immune splenocytes are much less protected from VACV than recipients of whole splenocytes from LCMV-immune donors (Selin *et al.*, 1998).

Cross-reactive T cells are thought to be involved in immunoprotection against VACV. LCMV NP₂₀₅₋₂₁₂, GP₃₄₋₄₁, GP₁₁₈₋₁₂₅ and VACV A11R₁₉₈₋₂₀₅ are identified as cross-reactive between LCMV and VACV (Cornberg *et al.*, 2010; Kim *et al.*, 2005). Upon VACV infection, the cross-reactive LCMV-specific CD8 T cells greatly increase in number, although the magnitude of the response may vary between mice due to the

private specificity of the individual mouse (Kim *et al.*, 2005). A11R₁₉₈₋₂₀₅ cell lines from LCMV-immune mice can bind to both VACV A11R₁₉₈₋₂₀₅ and LCMV GP₁₁₈₋₁₂₅ or GP₃₄₋₄₁ tetramers, proliferate in response to VACV infection *in vivo*, and reduce VACV viral load in naïve recipients (Cornberg *et al.*, 2010).

There are biological differences between the VACV and LCMV infections. VACV replicates preferentially in peripheral organs like the ovaries while LCMV replicates primarily in the lymphoid organs. IFN γ inhibits VACV replication (Harris *et al.*, 1995; Karupiah *et al.*, 1993; Liu *et al.*, 2004), and the frequency of IFN γ -producing memory CD8 T cells correlate directly with protection against VACV (Moutaftsi *et al.*, 2009), but LCMV is not as sensitive to IFN γ (van den Broek *et al.*, 1995). LCMV is controlled by contact-dependent perforin-mediated cytotoxicity, but perforin or Fas have little role in the clearance of VACV (Kägi *et al.*, 1995; Walsh *et al.*, 1994). Therefore, I investigated what kinds of memory T cells are being generated in VACV- and LCMV-infected mice. Since it has been shown in homologous LCMV infection that the number of cytolytic CD8 T cells correlates directly with target killing (Ganusov *et al.*, 2011), I also examined the relationship between number of cross-reactive T cells and heterologous immunoprotection in VACV-challenged LCMV-immune mice.

Results

Memory CD8 T cells from VACV-GP-immune mice display phenotypes of stronger proliferative recall potential

Memory T cells of different qualities can be generated by different infections or vaccinations due to the different cytokine environments and antigen abundance. If these memory T cells were to be re-activated during subsequent infection, they may influence the host response and disease outcome differently. To see if memory CD8 T cells generated from LCMV and VACV infections are different in quality, I adoptively transferred P14 splenocytes containing $1-3 \times 10^4$ CD8 T cells into C57BL/6 mice and inoculated the recipients i.p. with either 5×10^4 pfu LCMV strain Armstrong or 5×10^6 pfu recombinant VACV containing LCMV glycoprotein (VACV-GP) on the same day. Six weeks or more later, P14 memory CD8 T cells were analyzed from the spleen using their congenic marker Thy1.1 in phenotypic and functional assays so that the same transgenic T cells being activated in the context of two different infections were examined.

Killer cell lectin-like receptor G1 (KLRG1) signaling in CD8 T cells impairs proliferation, and its expression correlates with proliferative incapacity of antigen-experienced CD8 T cells (Gründemann *et al.*, 2006; Voehringer *et al.*, 2001). CD127 is important for homeostatic proliferation of memory CD8 T cells (Schluns *et al.*, 2000). The combination of KLRG1 and CD127 has been used to classify memory CD8 T cells: CD127^{hi} KLRG1^{lo} as long-term memory T cells, CD127^{hi} KLRG1^{hi} as effector memory T cells, and CD127^{lo} KLRG1^{hi} as terminally differentiated effectors (Belz & Kallies, 2010). In both LCMV- and VACV-GP-immune hosts, over 50% of the memory

P14 CD8 T cells showed a long-term memory phenotype (CD127^{hi} KLRG1^{lo}) at 7 weeks post infection (Fig. 7.1a). A higher proportion of P14 memory CD8 T cells from LCMV-immune mice was KLRG1^{hi} (both 127^{hi} and 127^{lo}), suggesting that LCMV-immune memory T cells may have a lower proliferative potential. The phenotype of the memory population changed over time, and over 90% of P14 memory CD8 T cells from both immune groups displayed the long-term memory phenotype at 23 weeks post infection (data not shown).

Activation marker expression pattern on memory CD8 T cells has been shown to correlate with recall response potential in terms of accumulation at the site of infection and IL-2 production (Hikono *et al.*, 2007), and the phenotypes of cells exhibiting recall response potential from high to low are as follows: CD27^{hi} CD43^{lo} > CD27^{hi} CD43^{hi} > CD27^{lo} CD43^{lo}. In LCMV-immune mice, there was a lower frequency of P14 memory CD8 T cells that expressed the higher recall potential phenotype (CD27^{hi} CD43^{lo}), and about 50% expressed the lower recall potential phenotype (CD27^{lo} CD43^{lo}) at 6 weeks post infection (Fig. 7.1b). On the other hand, about 60% of P14 memory CD8 T cells from VACV-GP-immune mice expressed the higher recall potential phenotype and relatively small percentage of cells were of the lower recall potential phenotype. Both groups had a relatively low percentage of CD27^{hi} CD43^{hi} P14 memory CD8 T cells, but over time, the frequency of these cells would gradually increase while the frequency of lower recall potential P14 memory CD8 T cells would decline. At 23 weeks post infection, a higher percentage of the CD27^{hi} CD43^{lo} P14 memory CD8 T cells was found in LCMV-immune mice (data not shown).

The P14 memory CD8 T cells from LCMV- and VACV-GP-immune hosts are phenotypically different. The analysis by surface markers suggested that memory T cells from VACV-GP-immune mice were more capable of recall responses and should theoretically have the capacity to protect against subsequent LCMV infections.

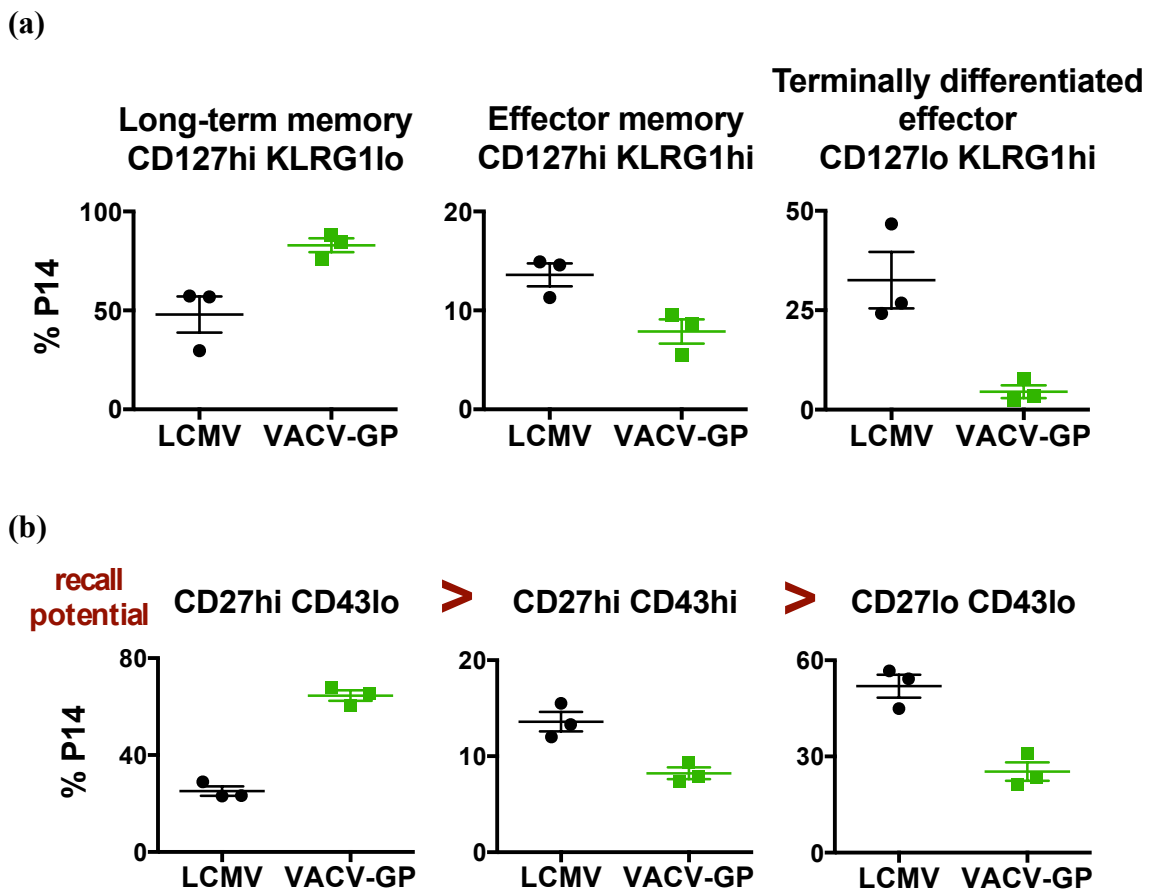


Figure 7.1. LCMV and VACV-GP infections generate memory CD8 T cells of different phenotype compositions. C57BL/6 mice received Thy1.1 P14 splenocytes were infected i.p. with 5×10^4 pfu LCMV or 5×10^6 pfu recombinant VACV-GP. Splenocytes were analyzed for (a) CD127 and KLRG1 expression at week 7 post infection, and for (b) CD27 and CD43 expression at week 6 post infection. Data are representative of at least two separate experiments at similar timepoints.

More memory CD8 T cells from LCMV-immune mice are multiple-cytokine producers

To evaluate the effector functions of the memory CD8 T cells, splenocytes from LCMV- and VACV-GP-immune mice were examined by ICS after *in vitro* stimulation with GP₃₃₋₄₁ synthetic peptide. The majority of Thy1.1 P14 memory CD8 T cells from both immune groups were able to produce IFN γ and TNF upon peptide stimulation (Fig. 7.2). A greater proportion of P14 memory CD8 T cells from LCMV-immune mice than those from VACV-GP-immune mice produced three cytokines, IFN γ , TNF and IL-2, in the same cells. More P14 memory CD8 T cells from VACV-GP-immune mice produced IFN γ alone when stimulated. The cytokine production profile revealed that LCMV infection generated memory CD8 T cells of more versatile effector functions.

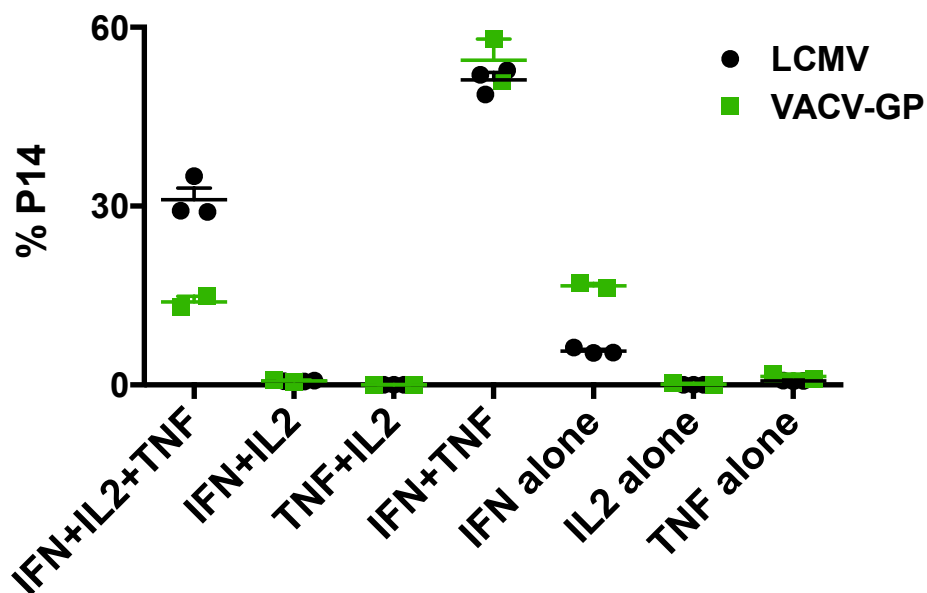


Figure 7.2. Greater proportion of memory CD8 T cells from LCMV infection is capable of producing multiple cytokines in the same cells upon re-stimulation. C57BL/6 mice receiving Thy1.1 P14 splenocytes were infected i.p. with 5×10^4 pfu LCMV or 5×10^6 pfu recombinant VACV-GP. Splenocytes harvested at day 46 (weeks 6.5) post infection were analyzed for cytokine production by ICS after GP₃₃₋₄₁ peptide stimulation for 5 hr. Data are representative of three separate experiments.

Memory CD8 T cells from both immune groups proliferate and produce granzyme B upon stimulation in vitro

To compare the proliferative responses to re-stimulation *in vitro*, CFSE-labeled splenocytes from LCMV- and VACV-GP-immune mice were stimulated with GP₃₃₋₄₁ peptide in a 96-well plate for 4-6 days. The frequency of Ly5.1 P14 memory CD8 T cells was higher in the LCMV-immune host than in the VACV-GP-immune host. Therefore, using the frequency determined for each mouse prior to CFSE-labeling, the P14 memory CD8 T cells were plated in equal numbers along with supplementary naïve splenocytes to make up equal total number of cells in each well. After peptide stimulation, P14 memory CD8 T cells from both LCMV- and VACV-GP-immune mice proliferated, and the frequencies of CFSE-diluted P14 memory CD8 T cells in both immune groups were above 80% by day 6 of *in vitro* stimulation (Fig. 7.3a).

Upon re-stimulation, memory cells produce granzyme in vesicles in preparation for target cell killing. The accumulation of lytic granules in memory CD8 T cells was evaluated along with the *in vitro* proliferation experiment. Peptide stimulation caused similar proportions of P14 memory CD8 T cells from the LCMV- and the VACV-GP-immune mice to produce granzyme B after 4 days (Fig. 7.3b). At day 6 post stimulation, over 80% of P14 memory CD8 T cells in both groups have accumulated granzyme B.

In contrast to the different marker phenotypes and cytokine profiles, P14 memory CD8 T cells from LCMV- and VACV-GP-immune hosts responded to re-stimulation with similar levels of proliferation and granzyme B accumulation *in vitro*.

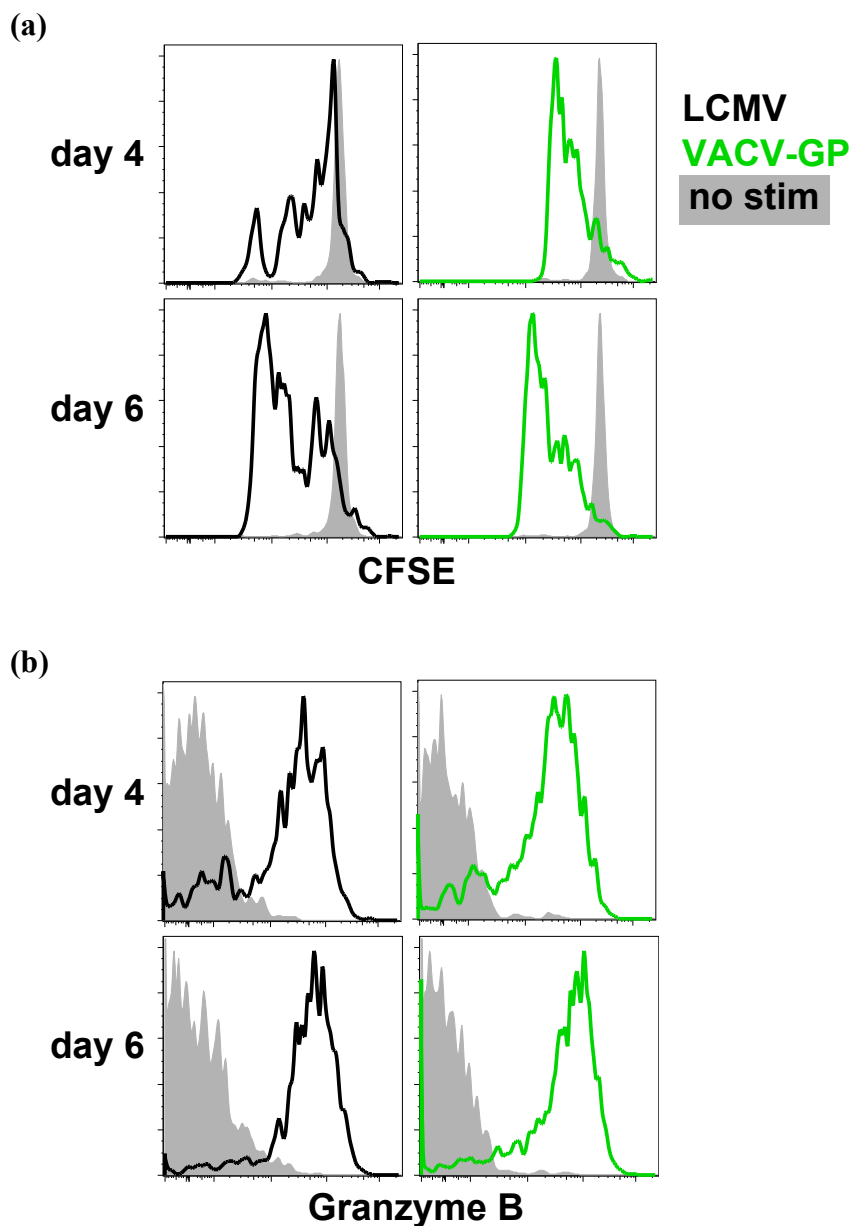


Figure 7.3. P14 memory CD8 T cells from LCMV- and VACV-GP-immune mice proliferate and accumulate granzyme B upon *in vitro* stimulation. C57BL/6 mice the received Ly5.1 P14 splenocytes were infected i.p. with 5×10^4 pfu LCMV or 5×10^6 pfu recombinant VACV-GP. Splenocytes harvested at day 85 (week 12) post infection were CFSE-labeled and stimulated with GP₃₃₋₄₁ peptide for 4-6 days. The numbers of Ly5.1 P14 memory CD8 T cells were equalized for each well using supplementary naïve splenocytes. Cells were analyzed for (a) proliferation and (b) granzyme B accumulation. Data are representative of two separate experiments.

Greater number of potentially cross-reactive memory CD8 T cells in LCMV-immune mice

The magnitude of a T cell response is influenced by the precursor frequency, and the proliferative response may determine the outcome of infections. If cross-reactive memory CD8 T cells mediate heterologous immunoprotection between LCMV and VACV, the number of potentially cross-reactive memory CD8 T cells in the immune mice may be critical. On average, there were about 1.2×10^4 A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in a VACV-immune mouse per spleen (Table 7.1). The virus-specific memory CD8 T cell frequency was generally higher in the LCMV-immune mice. Combining the memory CD8 T cell populations that recognize the three cross-reactive epitopes GP₃₄₋₄₁, GP₁₁₈₋₁₂₅ and NP₂₀₅₋₂₁₂, there are approximately 1.2×10^5 potentially cross-reactive CD8 T cells per LCMV-immune spleen. However, due to the phenomenon of private specificity, only a fraction of these cells may actually cross-react and respond during heterologous infection. Nevertheless, there are still 10 times more memory CD8 T cells that may potentially cross-react with VACV in the LCMV-immune mice. The lower number of potentially cross-reactive memory CD8 T cells may be one of the reasons why a history of VACV infection is not protective during subsequent LCMV infection.

Table 7.1. LCMV-immune mice possess a greater number and frequency of memory CD8 T cells that are specific for the cross-reactive epitopes between LCMV and VACV. C57BL/6 mice were inoculated with 5×10^4 pfu LCMV Armstrong or 10^6 pfu VACV. The frequency of epitope-specific memory CD8 T cells were determined in the spleen of the immune mice after at least 8 weeks post infection by ICS.

Specificity:	VACV-immune	LCMV-immune		
	A11R₁₉₈₋₂₀₅	GP₃₄₋₄₁	GP₁₁₈₋₁₂₅	NP₂₀₅₋₂₁₂
	17795 (0.09%)	147362 (1.0%)	22956 (0.15%)	30442 (0.20%)
	20258 (0.16%)	45323 (0.49%)	10277 (0.11%)	14194 (0.15%)
	5164 (0.06%)	50350 (0.5%)	25229 (0.26%)	35223 (0.35%)
	8019 (0.09%)	30518 (0.77%)	16436 (0.41%)	5712 (0.14%)
	8199 (0.09%)	93536 (1.2%)	38985 (0.50%)	22293 (0.29%)
Average:	11887 (0.1%)	73418 (0.8%)	22776 (0.3%)	21573 (0.2%)

DC(A11R)-immunization boosts A11R-specific memory and confers heterologous immunoprotection

To examine whether the number of potentially cross-reactive memory CD8 T cells is critical for protection during heterologous infection between LCMV and VACV, I attempted to increase the number of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in a VACV-immune mice by DC-immunization and tested whether the higher number of cross-reactive memory CD8 T cells may confer protection against LCMV. Immortalized DC2.4 cells were pulsed with A11R₁₉₈₋₂₀₅ peptide, irradiated, and administered subcutaneously (s.c.). Seven days later, pulsed DC-immunized and mock-immunized (HBSS-injected) mice were infected i.p. with 10⁶ pfu VACV. After 6 weeks, mice were screened for A11R₁₉₈₋₂₀₅-specific memory CD8 T cell frequency in the blood. Peptide-pulsed DC-priming greatly increased the frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in the VACV-immune mice (Fig. 7.4a). The frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in VACV-immune mice was less than 0.1%, and DC-immunization without VACV infection did not generate appreciable A11R₁₉₈₋₂₀₅-specific memory CD8 T cells.

Immune mice were subsequently challenged i.p. with 5x10⁴ pfu LCMV Armstrong. Viral titer and virus-specific T cell response were evaluated at 6 days post LCMV infection. Protection was determined for each experiment separately. Mice were considered protected when their LCMV titers in the spleen at day 6 post infection were lower than three times the standard deviation below, and at least half a log below, the geometric mean LCMV titer in the VACV-immune control group. In the DC-immunized

VACV-immune group, some of the mice were protected and had lower LCMV titers at day 6 post infection (Fig. 7.4b).

Among the DC-immunized VACV-immune mice that achieved a higher A11R₁₉₈₋₂₀₅-specific memory CD8 T cell frequency, only some of them reduced LCMV titer after challenge (Fig. 7.4c). The elevated A11R₁₉₈₋₂₀₅-specific memory CD8 T cell frequency seemed to contribute to the protection against LCMV infection because none of the VACV-immune mice, whose A11R₁₉₈₋₂₀₅-specific memory CD8 T cell frequency was low, reduced LCMV titer. However, a high pre-bleed frequency did not guarantee immunoprotection from LCMV challenge. The phenomenon of private specificity probably plays an important role in governing which immune T cell population can respond to the cross-reactive LCMV epitopes and protect against heterologous LCMV infection.

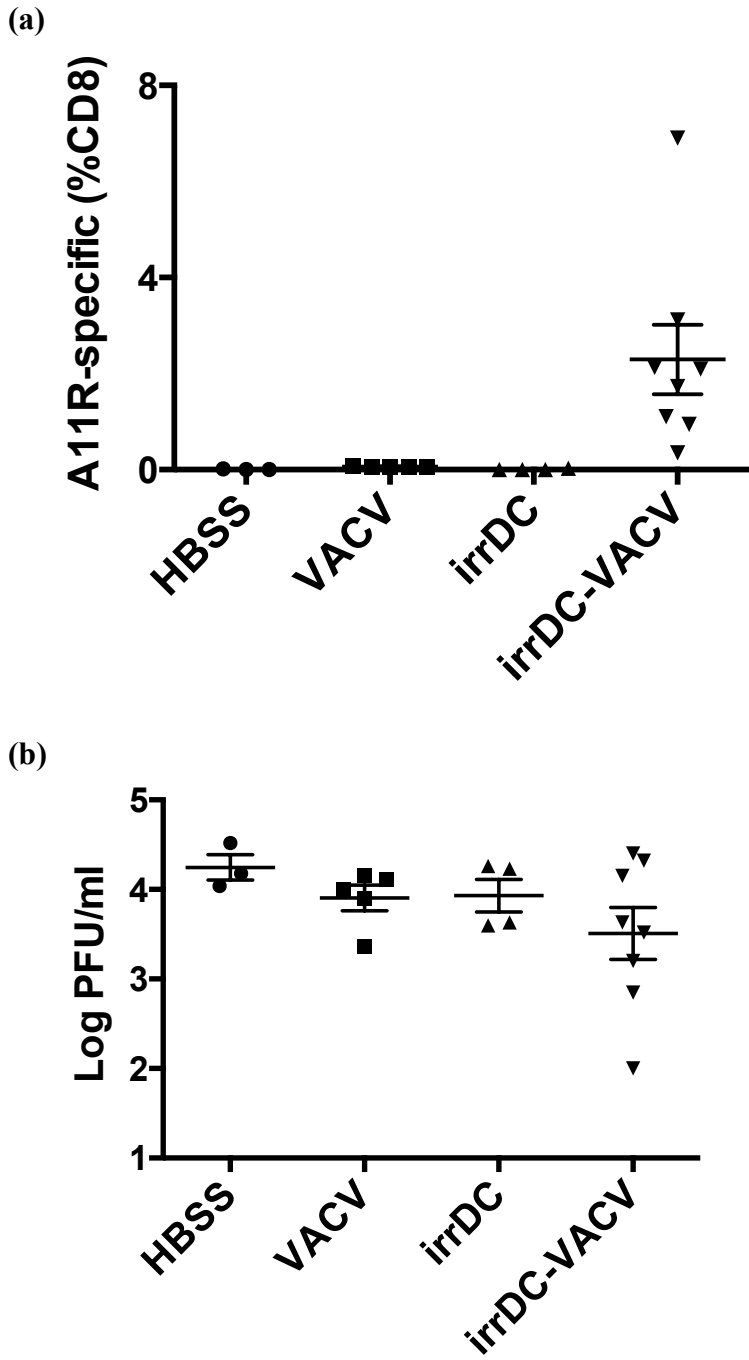


Figure 7.4.

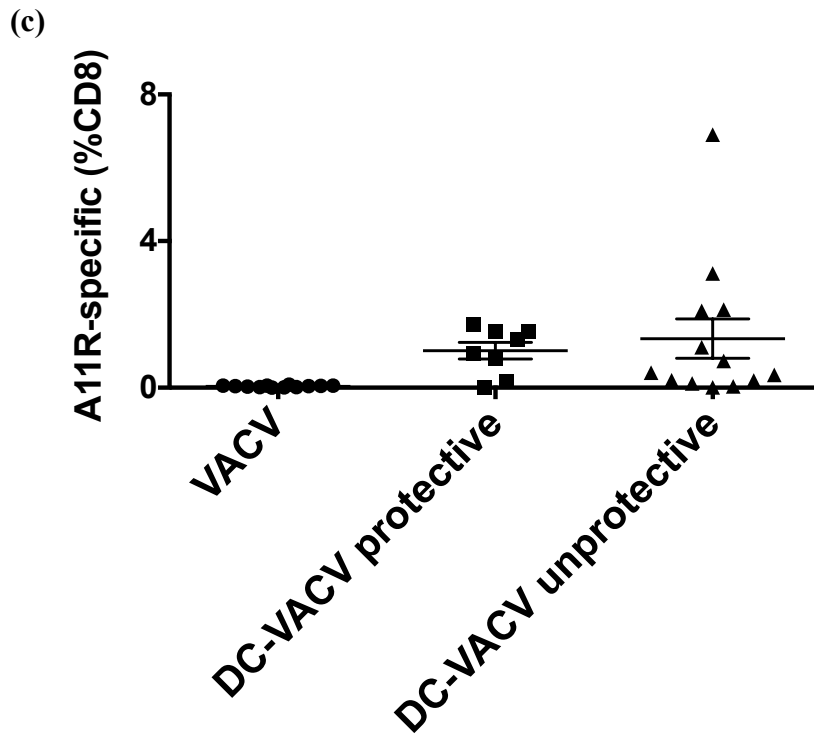


Figure 7.4. Immunization with irradiated A11R₁₉₈₋₂₀₅-pulsed DC2.4 greatly increases the frequency of A11R₁₉₈₋₂₀₅-specific CD8 T cells in VACV-immune mice and correlates with protection of some mice during heterologous LCMV challenge. C57BL/6 mice were immunized s.c. with A11R₁₉₈₋₂₀₅-pulsed irradiated DC2.4 and infected i.p. with 10⁶ pfu VACV after 7 days. (a) At week 6 post VACV infection, the frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells was measured in blood by ICS before i.p. challenge with 5x10⁴ pfu LCMV Armstrong. (b) LCMV titer was determined in the spleen at day 6 post heterologous challenge. Mice with LCMV titers lower than three times the standard deviation below, and at least half a log below, the geometric mean LCMV titer in the VACV-immune control group were considered protected. (c) Combined data from four similar experiments comparing LCMV titer at day 6 post infection to frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in blood prior to LCMV challenge suggested that high pre-bleed A11R₁₉₈₋₂₀₅-specific CD8 T cell frequency could not guarantee immunoprotection during heterologous LCMV challenge.

Virus-specific CD8 T cell response correlates with protection against LCMV

The frequency and number of cross-reactive T cells may change when the T cells respond to a subsequent heterologous infection. Comparing the A11R₁₉₈₋₂₀₅-specific memory CD8 T cell frequency after background subtraction (net frequency) at day 6 post LCMV challenge between mice that were protected during LCMV infection and those that were not within the DC-immunized VACV-immune group, a threshold of 0.59% of CD8 T cells was found to correlate with protection against LCMV infection (Fig. 7.5a). The segregation was more obvious when all mice regardless of their history of infection were included in the analysis (Fig. 7.5b). The animals that were protected during LCMV infection tend to have 0.59% or higher frequency of CD8 T cells that were A11R₁₉₈₋₂₀₅-specific at day 6 post LCMV infection.

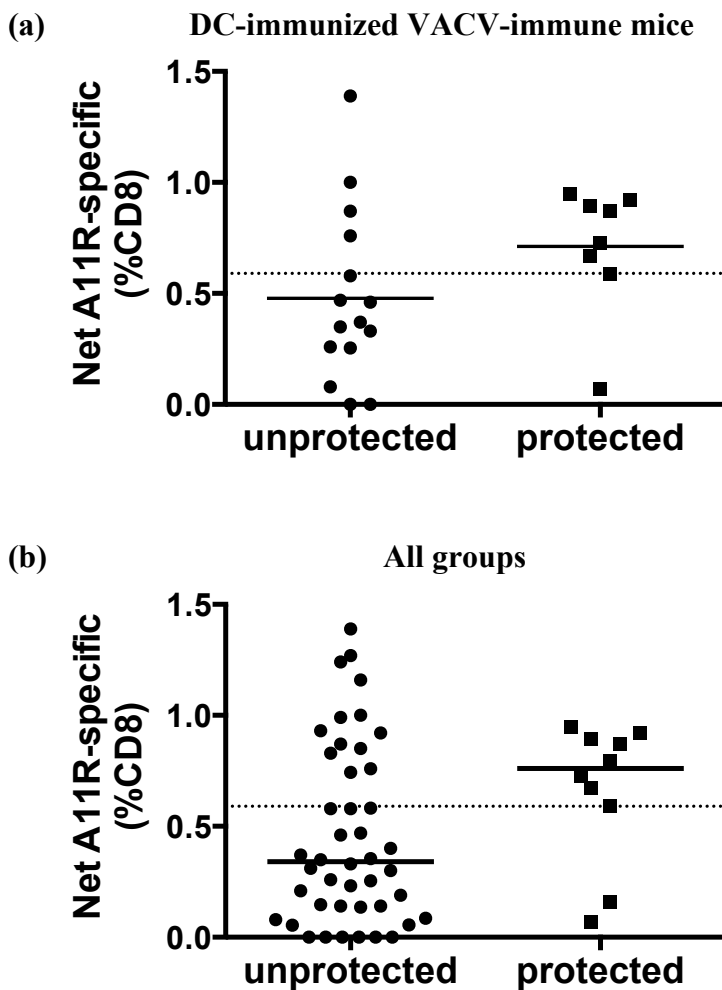


Figure 7.5. A11R₁₉₈₋₂₀₅-specific CD8 T cell frequency of at least 0.59% at day 6 post LCMV infection correlates with protection. DC-immunized VACV-immune mice and controls were infected i.p. with 5×10^4 pfu LCMV. LCMV viral titer in the spleen and A11R₁₉₈₋₂₀₅-specific CD8 T cell frequency were evaluated at 6 days post LCMV infection. Mice with LCMV titers lower than three times the standard deviation below, and at least half a log below, the geometric mean LCMV titer in the VACV-immune control group were considered protected. Net A11R₁₉₈₋₂₀₅-specific CD8 T cell frequency in the spleen between the protected and the unprotected animals in (a) DC-immunized VACV-immune group ($p = 0.0272$) and (b) all groups ($p = 0.0107$) were analyzed by Fisher's exact test. Dotted lines in each graph marked the 0.59% threshold frequency.

The relative proportional response to the major LCMV-specific epitopes was calculated from the CD8 T cell responses to GP₃₄₋₄₁, GP₁₁₈₋₁₂₅, NP₂₀₅₋₂₁₂, NP₃₉₆₋₄₀₄ and GP₂₇₆₋₂₈₆ at day 6 post LCMV infection. DC-immunized VACV-immune mice that were protected during LCMV infection mounted a significantly higher proportional response to NP₃₉₆₋₄₀₄ than the VACV-immune control mice did (Fig. 7.6a), and they displayed a significantly lower proportional response to NP₂₀₅₋₂₁₂ and GP₁₁₈₋₁₂₅ (Fig. 7.6b), while the proportional responses to GP₃₄₋₄₁ and GP₂₇₆₋₂₈₆ were not significantly different between groups (Fig. 7.6c). In this regard, the response of the DC-immunized VACV-immune mice that were not protected during LCMV infection resembled that of the VACV-immune mice, which were similarly not protected during LCMV infection. Higher viral loads may have caused exhaustion of the NP₃₉₆₋₄₀₄-specific CD8 T cells, and in these experiments some mice would produce less TNF and mount a lower CD8 T cell response than others. However, the occurrence of partial exhaustion was rare, and was not particularly associated with the group of mice that were not protected during LCMV infection. On the other hand, the adoptively transferred NP₃₉₆₋₄₀₄-specific CD8 T cell line has been shown to be the most effective, in comparison to the GP₂₇₆₋₂₈₆- and GP₃₃₋₄₁-specific CD8 T cell lines, in clearing LCMV infection in mice (Gallimore *et al.*, 1998), consistent with the observation here that the higher NP₃₉₆₋₄₀₄-specific proportional response correlated with better protection.

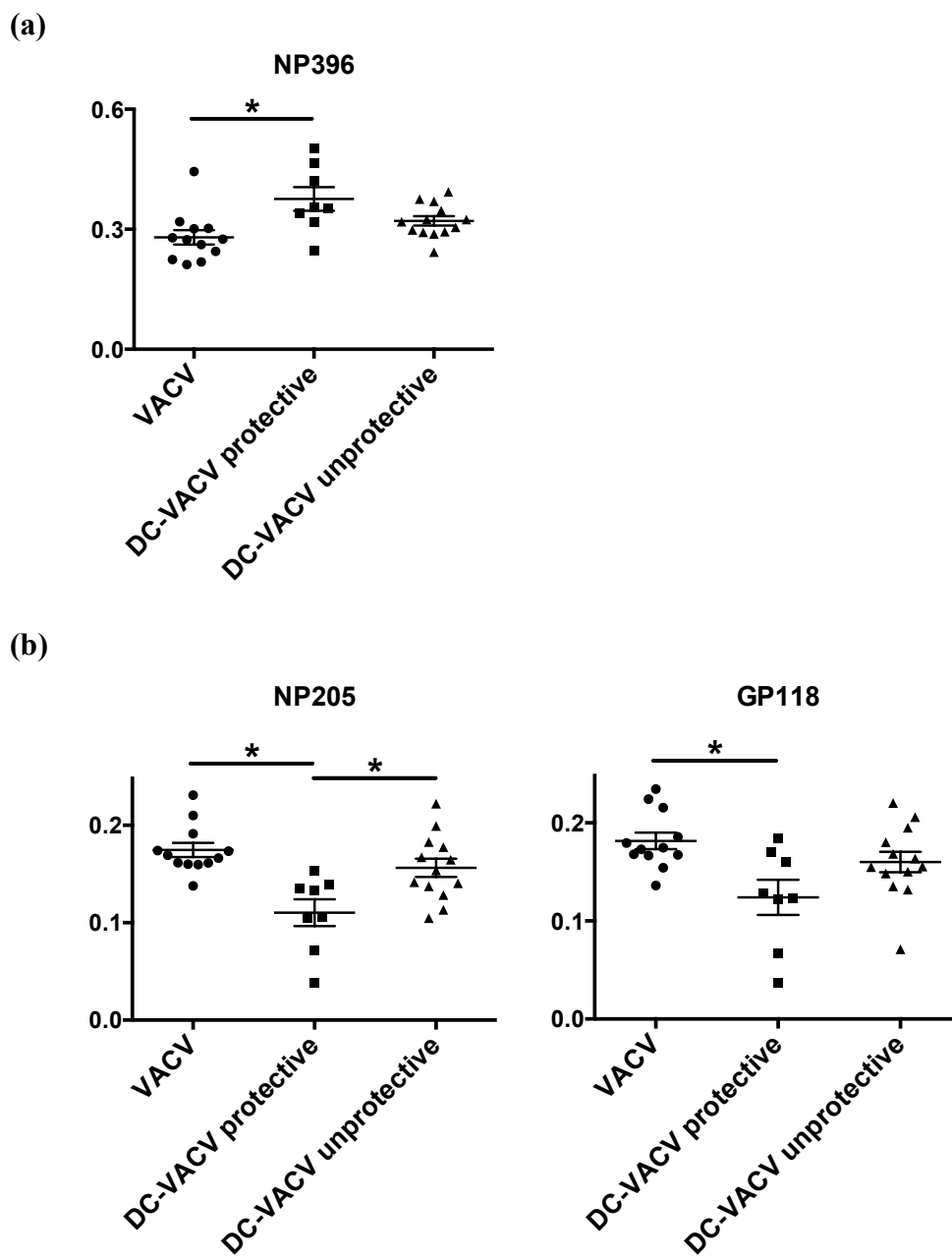


Figure 7.6.

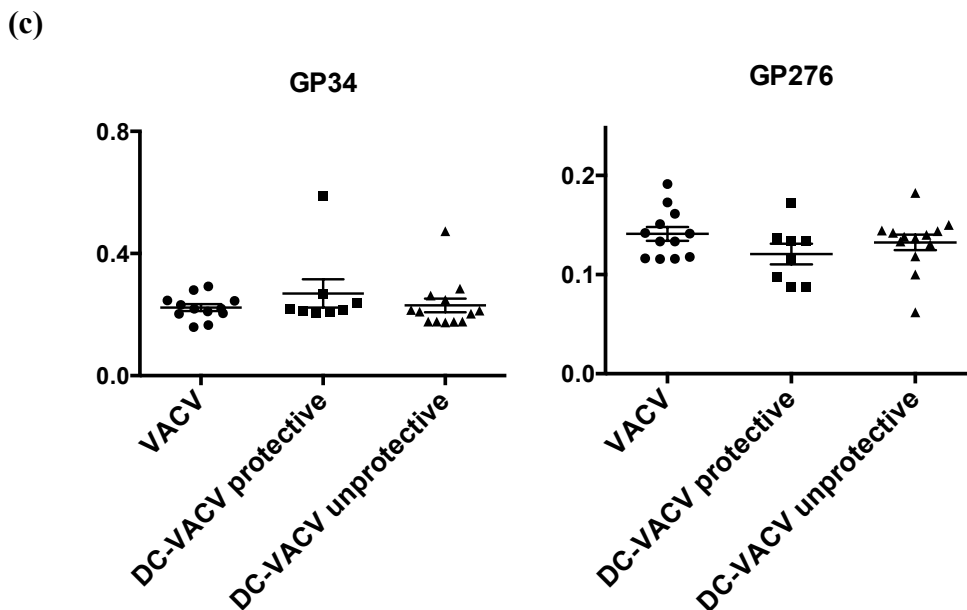


Figure 7.6. Proportional responses to LCMV-specific epitopes show significant difference between DC-immunized VACV-immune and VACV-immune mice. DC-immunized VACV-immune mice were infected i.p. with 5×10^4 pfu LCMV. Proportional responses to LCMV epitopes were calculated from CD8 T cells responding to GP₃₄₋₄₁, GP₁₁₈₋₁₂₅, NP₂₀₅₋₂₁₂, NP₃₉₆₋₄₀₄ and GP₂₇₆₋₂₈₆ at day 6 post LCMV infection. DC-immunized VACV-immune mice protected from LCMV infection displayed a significantly higher proportional response to NP₃₉₆₋₄₀₄ (a) but lower proportional responses to NP₂₀₅₋₂₁₂ and GP₁₁₈₋₁₂₅ (b). Proportional responses to GP₃₄₋₄₁ and GP₂₇₆₋₂₈₆ were not significantly different between groups (c). (*) denotes statistical significance ($p < 0.05$) between groups.

Discussion

Heterologous immunity, the immunity that can be developed to one pathogen after a host had been exposed to a different unrelated pathogen, is not necessarily reciprocal. A history of LCMV infection protects mice during VACV infection but a history of VACV infection does not protect against LCMV. Cross-reactive CD8 T cells from LCMV-immune mice have been shown to mediate heterologous protection against VACV (Cornberg *et al.*, 2010). By increasing the number of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells, here I showed that the number of cross-reactive CD8 T cells can influence the outcome of heterologous infection, implying that the low frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells in VACV-immune mice may be part of the reason why a history of VACV infection does not protect against LCMV infection.

The memory CD8 T cells generated from LCMV and VACV infections were phenotypically different, but the phenotypic differences failed to explain the non-reciprocity of heterologous immunoprotection. The phenotype of the VACV B8R₂₀₋₂₇-specific memory CD8 T cells from VACV-GP- and WT VACV-immune mice were similar, with comparable frequencies of multiple cytokine-producing cells and similar composition of cells of different recall potential phenotypes by their CD27 and CD43 expressions (data not shown). Therefore, the phenotypes of the memory CD8 T cells generated from LCMV- and VACV-GP-infected mice were compared using adoptively transferred P14 CD8 T cells with a congenic marker so that the memory generated in the same pool of cells in two different infection environments might be compared. The observation that LCMV infection generated more memory CD8 T cells that produce

multiple cytokines in the same cells may explain why LCMV-specific CD8 T cells are efficient in eliminating VACV-infected targets. However, the memory CD8 T cells generated by VACV-GP infection displayed a phenotype of higher recall potential (CD27^{hi} CD43^{lo}) and little proliferative incapacity (KLRG1^{lo}), suggesting their readiness to respond to subsequent infections, and contradicting their inability to reduce LCMV viral titer. Further experiments showed that the memory CD8 T cells from both LCMV- and VACV-GP-immune hosts could proliferate and produce granzyme B upon *in vitro* re-stimulation, ultimately arguing against any relationship between the phenotypic differences in the memory CD8 T cells generated from the two infections and the non-reciprocity of heterologous immunoprotection between LCMV and VACV.

Cross-reactive LCMV-specific memory CD8 T cells can respond to subsequent heterologous VACV infection, and so can cross-reactive VACV-specific memory CD8 T cells to LCMV infection. It has been shown that the cross-reactive T cells from LCMV-immune mice can proliferate during acute VACV infection (Kim *et al.*, 2005), but LCMV only minimally elicits proliferation of VACV-specific memory T cells at day 6 post infection (Kim *et al.*, 2002). The low frequency of the cross-reactive memory CD8 T cells in the VACV-immune mice probably made the detection of their proliferation difficult. However, a more specific focus on the A11R₁₉₈₋₂₀₅-specific CD8 T cells revealed their proliferative response upon LCMV infection. When CFSE-labeled whole splenocytes from VACV-immune mice were adoptively transferred into naïve congenic hosts, which were subsequently infected with LCMV, only 0.5% of the donor splenocytes were found to be A11R₁₉₈₋₂₀₅-specific by ICS at day 6 post LCMV infection, but all of the

A11R₁₉₈₋₂₀₅-specific CD8 T cells were CFSE low (data not shown), suggesting that the A11R₁₉₈₋₂₀₅-specific memory CD8 T cells had proliferated during LCMV infection. Moreover, the expanded cross-reactive T cells can be detected when T cells return to homeostasis. In VACV-LCMV double immune mice, the number of cross-reactive A11R₁₉₈₋₂₀₅-specific memory CD8 T cells at 9 weeks post LCMV infection was on average 10 times higher than the number in VACV-immune mice (Fig. 4.7 in Chapter IV). If antigens from both LCMV and VACV can activate the cross-reactive memory T cells and cause them to proliferate upon heterologous infection, the cross-reactive memory T cells should theoretically be protective regardless of the sequence of infections. However, the number of cross-reactive memory CD8 T cells may be much lower in the VACV-immune mice than in the LCMV-immune mice. The limited number of proliferated cross-reactive memory CD8 T cells may not make any difference in protecting VACV-immune mice during LCMV infection, since LCMV infection can by default elicit a strong CD8 T cell response in naïve mice.

In this study, I show that the number of cross-reactive memory CD8 T cells can influence the outcome of heterologous infection. A11R₁₉₈₋₂₀₅-pulsed DC-immunization greatly increased the frequency of A11R₁₉₈₋₂₀₅-specific CD8 T cells in VACV-immune mice. Although the frequency of A11R₁₉₈₋₂₀₅-specific CD8 T cells before LCMV challenge did not predict or correlate with protection, the net frequency (after background subtraction) of A11R₁₉₈₋₂₀₅-specific CD8 T cells at day 6 post LCMV infection did. This is consistent with the phenomenon of private specificity, where not every T cell population recognizing a cross-reactive epitope in one virus will react with the cross-

reactive epitope in the cross-reactive virus. Therefore, a high frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells before LCMV challenge may not predict protection. It has been shown that a certain number of T cells are required to eliminate the infected targets and control viral spread. Calculation from a previous study suggested that 10^4 LCMV-specific effector CD8 T cells could reduce virus titer by 1.5 log and 10^5 cells could eliminate the virus in the first 20 hrs post inoculation (Ehl *et al.*, 1997). Either the pre-existing virus-specific memory T cells or their progeny can meet the required T cell number early during infection. Factoring in the probability of private response and the different avidity of interaction between the infected targets and the A11R₁₉₈₋₂₀₅-specific memory CD8 T cells, the cutoff at 0.59% CD8 T cells of A11R₁₉₈₋₂₀₅-specificity that correlated with LCMV titer reduction falls within the range of that previous calculation. Although this cutoff frequency does not have any predictive value, it shows that the protection by a relatively higher frequency of A11R₁₉₈₋₂₀₅-specific memory CD8 T cells at day 6 post LCMV infection is not a random event. Therefore, both private specificity and the number of cross-reactive memory CD8 T cells determine the outcome of heterologous infection between LCMV and VACV.

Further analysis revealed that the higher proportional response to NP₃₉₆₋₄₀₄ at day 6 post LCMV infection correlated with better protection. Although NP₃₉₆₋₄₀₄ is not as cross-reactive as NP₂₀₅₋₂₁₂, GP₃₄₋₄₁ or GP₁₁₈₋₁₂₅ in LCMV-immune mice after VACV challenge (Kim *et al.*, 2005), NP₃₉₆₋₄₀₄-specific memory CD8 T cell frequency is often higher in the LCMV-VACV and VACV-LCMV double immune mice than in the LCMV-immune mice (Fig. 4.9 in Chapter IV and data not shown). Since the adoptively

transferred NP₃₉₆₋₄₀₄-specific CD8 T cell line is the most sensitive to its cognate antigen and the most effective in lysing target cells *in vitro* and clearing LCMV infection *in vivo* (Gallimore *et al.*, 1998), it is conceivable that the higher proportional response to NP₃₉₆₋₄₀₄ would correlate with better protection against LCMV infection. The A11R₁₉₈₋₂₀₅-specific memory CD8 T cells may have cross-reacted with and caused a relatively higher proportional response to NP₃₉₆₋₄₀₄, NP₂₀₅₋₂₁₂, GP₃₄₋₄₁ or GP₁₁₈₋₁₂₅ according to the private specificity of the memory CD8 T cell repertoire, but the mice attaining a higher proportional response to NP₃₉₆₋₄₀₄ were relatively more protected.

Chapter VIII. Discussion

In this thesis, I studied the generation and stability of CD8 T cells in the context of viral infections and investigated the influence of the history of infections in subsequent heterologous infections. I found that the immunoproteasomes were important in determining the magnitude and diversity of CD8 T cell response and memory. Besides the presence of cross-reactive memory T cells, changes in the endopeptidase activities of the immunoproteasomes can also alter the immunodominance hierarchy of CD8 T cell response. Memory CD8 T cells were previously shown to be reduced after heterologous infections. In this study, I re-examined the phenomenon, and additionally I showed that memory CD44hi CD4 T cells were also reduced by heterologous infections. In contrast to the susceptibility of memory CD44hi CD8 and CD44hi CD4 T cells to apoptosis and attrition, I found that Treg cells were more resistant to virus infection-induced apoptosis, and were more stable in number than CD44hi CD8 and CD44hi CD4 T cells early during LCMV infection.

The memory developed to one pathogen can influence the immune response to subsequent infections with unrelated pathogens and deviate the disease course and outcome. In this thesis, I examined the phenomenon of heterologous immunity in persistent MCMV infection, and found that LCMV-immune mice showed more severe immunopathology in the visceral fat pads early during MCMV infection, elevated viral titers, and fewer MCMV-specific inflationary memory CD8 T cells at 12 weeks post infection. Previously it has been established that the immunoprotection between LCMV and VACV is not reciprocal. As I studied the reasons behind this non-reciprocity of

heterologous immunity, I found that memory CD8 T cells generated from LCMV and VACV (VACV-GP) infections were phenotypically different, but the differences did not explain the non-reciprocity of the immunoprotection between LCMV and VACV. However, by increasing the cross-reactive A11R₁₉₈₋₂₀₅-specific memory CD8 T cells, more VACV-immune mice showed reduced titers on day 6 post LCMV challenge, and I found that protection correlated with higher net frequency of A11R₁₉₈₋₂₀₅-specific CD8 T cells and proportional response to NP₃₉₆₋₄₀₄ on day 6 post LCMV infection.

Shaping the CD8 T cell response and memory generation

Both the CD8 T cell response and memory CD8 T cell generation can be shaped by the immunoproteasomes. During infections, T cells “see” the pathogens through antigen presentation by MHC molecules. Both the T cell repertoire and the peptides presented can determine the magnitude and breadth of the T cell response. Immunoproteasomes are instrumental in generating peptides for presentation on MHC class I molecules, and their expression in the medullary thymic epithelial cells (Nil *et al.*, 2004) also implies their role in T cell selection. In Chapter III, I showed that mice genetically lacking all three immunoproteasome subunits were greatly impaired in their response to LCMV infection. The overall defect was much more severe than the sum of all defects previously reported in the singly deficient mice (Basler *et al.*, 2006; Nussbaum *et al.*, 2005). Antigen presentation in the triple KO mice was not as effective as the WT mice in activating the responding CD8 T cells, resulting in a lower percentage of activated CD8 T cells and thus a reduced response. Further analysis revealed a

differential impact of the deficiency of immunoproteasomes on different LCMV epitopes. GP₃₃₋₄₁ and GP₃₄₋₄₁ can probably be processed by both constitutive proteasomes and the immunoproteasomes and hence the GP₃₃₋₄₁- and GP₃₄₋₄₁-specific responses were reduced, but existent in the triple KO mice. However, GP₁₁₈₋₁₂₅ is completely dependent on the immunoproteasome for processing, and therefore the triple KO mice could not trigger any T cell response to GP₁₁₈₋₁₂₅. On the other hand, the reduced CD8 T cell responses to NP₂₀₅₋₂₁₂ and GP₂₇₆₋₂₈₆ were probably a combined result of the altered T cell repertoire and reduced presentation, while the T cell repertoire may have played a larger role in the response to GP₉₂₋₁₀₁, since the WT adoptively transferred T cells responded to GP₉₂₋₁₀₁ similarly in the WT hosts and the triple KO hosts. As a result, the immunodominance hierarchy is different in the triple KO and WT mice. The hierarchy of memory CD8 T cells largely reflected the acute CD8 T cell response to LCMV. Therefore, both the acute CD8 T cell response and memory generation were altered in the triple KO mice. The presentation by non-hematopoietic cells participates in the clonal expansion of CD8 T cells during LCMV infection (Thomas *et al.*, 2007), and therefore both constitutive proteasome and immunoproteasome subunits may influence the generation of CD8 T cell response and memory. MCMV has been shown to be dependent on the immunoproteasome for peptide processing, and the day 7 responses to M45₉₈₅₋₉₉₃, m141₁₆₋₂₃, m139₄₁₉₋₄₂₆ and M38₃₁₆₋₃₂₃ are greatly diminished in the LMP7 KO mice (Hutchinson *et al.*, 2011). I suspect that the triple KO may have a more deleterious effect on the acute response to MCMV infection.

Besides intrinsic factors like the T cell repertoire and antigen presentation, the history of infections may influence the immunodominance hierarchy of the T cell response. Pre-existing cross-reactive memory T cells expanding upon infections with the cross-reactive heterologous viruses can greatly shift the immunodominance hierarchy. It has been shown that the cross-reactive NP₂₀₅₋₂₁₂-specific CD8 T cells would dominate the T cell response to LCMV in PV-immune mice (Brehm *et al.*, 2002). In this study, I identified a novel potentially cross-reactive epitope pair between LCMV and MCMV. Approximately one in every five MCMV-immune mice responded exceedingly well to the otherwise subdominant LCMV epitope L₂₀₆₂₋₂₀₆₉, and shifted the immunodominance hierarchy with about 50% of the responding T cells being L₂₀₆₂₋₂₀₆₉-specific. A BLAST® search against the MCMV protein sequence identified M57₇₂₇₋₇₃₄, which can stimulate a large percentage of CD8 T cells from mice mounting a dominant response to LCMV L₂₀₆₂₋₂₀₆₉. LCMV L₂₀₆₂₋₂₀₆₉ and MCMV M57₇₂₇₋₇₃₄ share 50% sequence homology, and the rest of the sequences are biochemically similar, suggesting the same TCR might have recognized the two peptides through molecular mimicry (Hagerty & Allen, 1995; Welsh & Selin, 2002). M57₇₂₇₋₇₃₄ was not identified in the published epitope screen (Munks *et al.*, 2006b). I found in a preliminary experiment that both acutely MCMV-infected and long-term MCMV-immune mice responded to M57₇₂₇₋₇₃₄ at a very low frequency of around 0.2% or less CD8 T cells. This is similar to the low frequency response of VACV-infected mice to VACV A11R₁₉₈₋₂₀₅, which was also found by cross-reactivity to LCMV but did not come up in the epitope prediction screen (Kim *et al.*, 2005; Moutaftsi *et al.*, 2006).

Trimming the pre-existing memory populations by heterologous infections

Memory CD8 T cells are normally maintained in the periphery as a stable population by cytokines such IL-7 and IL-15 (Schluns *et al.*, 2000; Selin *et al.*, 1996; Tan *et al.*, 2002). Myeloid cells can also enhance CD8 T cell survival through co-stimulatory molecule CD70 signaling to CD27 on the CD8 T cells (Frasca *et al.*, 2010). However, this stability of CD8 T cell populations can be perturbed in some situations.

Heterologous viral infections can cause reduction of pre-existing memory CD8 T cells (Kim & Welsh, 2004; Liu *et al.*, 2003; Selin *et al.*, 1996; 1999). In Chapter IV, I re-examined the phenomenon in the resting epitope-specific memory CD8 T cells by ICS in infection sequences LCMV-MCMV, VACV-LCMV, VACV-LCMV-MCMV, VACV-MCMV, LCMV-VACV. Consistent with published data, I found that LCMV GP₃₃₋₄₁-, NP₃₉₆₋₄₀₄-, GP₂₇₆₋₂₈₆-, GP₁₁₈₋₁₂₅- and NP₂₀₅₋₂₁₂-specific memory CD8 T cells were significantly reduced after MCMV infection. Pre-existing VACV B8R₂₀₋₂₇- and E7R₁₃₀₋₁₃₇-specific memory CD8 T cells were also significantly reduced after LCMV infection. The reduced B8R₂₀₋₂₇-specific population by LCMV infection could be further reduced by subsequent MCMV infection. Although memory CD4 T cells spontaneously decline over time (Homann *et al.*, 2001), I showed that they can also be further reduced by heterologous infections. In this study, I showed that LCMV GP₆₁₋₈₀- and NP₃₀₉₋₃₂₈-specific memory CD4 T cells were reduced after MCMV infection, and also after VACV infection. Therefore, heterologous infections can reduce the number of both CD8 and CD4 memory T cells.

I found that persistent MCMV infection did not further erode pre-existing memory populations. The influence of persistent infection on T cell memory may vary depending on the nature of the infections. Chronic *Leishmania donovani* infection can promote the accumulation of CD8 T cells of central memory phenotype (Polley *et al.*, 2005), while chronic BCG and *Salmonella typhimurium* infections reduce pre-existing memory CD8 and CD4 T cells (Dudani *et al.*, 2008; Smith *et al.*, 2002). Persistent LCMV clone 13 infection has been shown to cause continuous attrition of pre-existing CD8 T cell memory even at 3 weeks post infection (Kim & Welsh, 2004); however, no continuous erosion of pre-existing memory was found in this study with an MCMV infection. It is possible that the cytokine environment in the MCMV-infected mice is more supportive for homeostatic survival of memory T cells while a low level of type I IFN in the LCMV clone 13-infected mice (Lee *et al.*, 2009) may promote apoptosis of memory T cells. Type I IFN is associated with early attrition of memory CD8 T cells (McNally *et al.*, 2001), and is also the third signal for clonal expansion of CD8 T cells especially after LCMV infection (Aichele *et al.*, 2006; Curtsinger *et al.*, 2005). The early memory attrition caused by type I IFN may make room for subsequent clonal expansion of the responding T cells and prevent pre-existing cross-reactive memory CD8 T cells from dominating a T cell response (Bahl *et al.*, 2006). The re-activation of MCMV genes can stimulate MCMV-specific memory inflation, but it probably does not elicit strong enough cytokine signals to reduce the pre-existing memory.

Heterologous infections can increase the number of cross-reactive memory T cells. Cross-reactive NP₂₀₅₋₂₁₂-specific memory CD8 T cells has been shown to increase

in LCMV-PV and PV-LCMV infection sequences (Brehm *et al.*, 2002). Proliferation of cross-reactive LCMV GP₃₃₋₄₁/GP₃₄₋₄₁⁻, GP₁₁₈₋₁₂₅⁻, NP₂₀₅₋₂₁₂, NP₃₉₆₋₄₀₄⁻, GP₂₇₆₋₂₈₆-specific CD8 T cells has been shown in LCMV-immune mice after VACV infection (Kim *et al.*, 2002; 2005). However, the cross-reactive VACV A11R₁₉₈₋₂₀₅-specific memory CD8 T cells persist at a very low frequency in VACV-immune mice, and therefore their proliferation during LCMV infection is very difficult to detect amidst the vigorous LCMV-specific T cell response. In Chapter IV of this thesis, I showed the expansion of A11R₁₉₈₋₂₀₅-specific memory CD8 T cell population in VACV-LCMV double immune mice when T cells returned to homeostasis. The proliferation of cross-reactive A11R₁₉₈₋₂₀₅-specific memory CD8 T cells was virus-specific because subsequent infection with MCMV, which does not cross-react with VACV, did not increase the A11R₁₉₈₋₂₀₅-specific memory CD8 T cells, but actually reduced the population. Because of the private specificity of T cell repertoires, the expansion of the A11R₁₉₈₋₂₀₅-specific memory CD8 T cells varied among the same group of mice with the same transgenic background. A similar phenomenon of private specificity was observed with the memory CD8 T cells specific for the novel potentially cross-reactive pair of epitopes identified between LCVM and MCMV. After LCMV infection, the frequency and number of MCMV M57₇₂₇₋₇₃₄-specific memory CD8 T cells was greatly increased in some MCMV-immune mice.

In Chapter IV, I showed that memory CD4 T cells were reduced by heterologous infections. In Chapter V, I also showed that early after LCMV infection, CD44^{hi} CD4 T cells were significantly reduced in number and became more apoptotic than their

uninfected counterparts, similar to CD44hi CD8 T cells previously reported (Bahl *et al.*, 2010). The reduction of memory CD8 T cells during viral infection is thought to be the result of apoptosis early during infection, in association with the peak of type I IFN at day 2 post infection (McNally *et al.*, 2001). Similar to memory CD44hi CD8 T cells, CD44hi CD4 T cells also undergo apoptosis after treatment with type I IFN inducer poly(I:C) (Bahl *et al.*, 2010). In parallel comparison with CD44hi CD8 T cells, the reduction of virus-specific memory CD4 T cells shown could also be the result of apoptosis and attrition of CD44hi CD4 T cells early during infection. A longitudinal study of epitope-specific memory CD4 T cells following a heterologous infection will be needed to confirm this.

Resistance of Treg cells to virus infection-induced apoptosis

In contrast, although Foxp3⁺ CD4⁺ Treg cells exhibit a partial memory phenotype with an intermediate to high expression of CD44 (Min *et al.*, 2007), I found that Treg cells are more resistant to early apoptosis than CD44hi memory CD8 and CD4 T cells, and were not significantly reduced after LCMV infection. Infections have been implicated as a potential trigger of autoimmune diseases (Huber, 2006; Sherbet, 2009; Van Der Werf *et al.*, 2007; Zipris *et al.*, 2003) but the underlying mechanism is still unknown. Transient reduction of Treg cell numbers can lead to the development of autoimmunity (Setoguchi *et al.*, 2005), leading us to suspect the role of infections in reducing the memory-like Treg cells and contributing to autoimmunity. However, the results in this study indicated that the Treg cells were more resistant to virus infection-

induced apoptosis, and the survival of Foxp3⁺ CD4⁺ Treg cells was not compromised early during infection. Studies have shown that Treg cells are able to survive insults from gamma-irradiation or topoisomerase inhibitors (Qu *et al.*, 2010; Winzler *et al.*, 2011), and Treg cells are resistant to activation-induced cell death (Banz *et al.*, 2002; Fritzsching *et al.*, 2005). Although the mechanism is not known, the survival of Treg cells seems to be regulated very differently from conventional CD4 T cells. Further studies will be needed to identify the differences in the cell death signaling pathways that will correlate with the susceptibility of memory CD44^{hi} CD8 and CD4 T cells and the relative resistance of Treg cells to virus infection-induced apoptosis observed in this work. Nonetheless, the resistance of Treg cells to apoptosis during infection may explain the low incidence of autoimmunity among infected patients. Although self-reactive antibodies can be generated during infections (Berlin *et al.*, 2007; Sane *et al.*, 2012; Sjöwall *et al.*, 2012), most patients survive viral infections without autoimmune consequences.

Treg cells are worth maintaining, even apart from the concern for autoimmunity development, because they play a critical role of regulating effector T cells, NK cells, neutrophils, monocytes, and macrophages early during viral infections (Bedoya *et al.*, 2013; Fulton *et al.*, 2010; Lund *et al.*, 2008; Veiga-Parga *et al.*, 2012). The presence of virus-specific memory Treg cells can preserve lung function by limiting CD8 T cell response and reducing pathology during homologous influenza re-challenge (Brincks *et al.*, 2013). However, in heterologous infections, the nature of their persistence may also pose a problem, as demonstrated in the influenza-immune mice during intranasal LCMV challenge, where the higher number of Treg cells contribute to more severe

immunopathology in the immune mice by preventing exhaustion of responding CD8 T cells and supporting migration of CD8 T cells into the lungs (Kraft *et al.*, 2013).

I found that Treg cells continue to express functional high-affinity IL-2 receptors on the cell surface and speculated that might provide a survival advantage to Treg cells during viral infections. Upon LCMV infection, the already low expression of CD127 was further reduced on Foxp3⁺ CD4⁺ Treg cells but the expression of CD25, CD122 and CD132 was either increased or unchanged. The IL-2 receptors were also functional, and IL-2 stimulation *in vitro* could instantly phosphorylate STAT5 in the Treg cells. It has been shown that the maintenance of Treg cells in the periphery depends on IL-2 secreted by other cells (Setoguchi *et al.*, 2005). The continuous expression of high-affinity IL-2 receptors on the cell surface might enable Treg cells to compete for IL-2 in the environment with other activated T cells during infections and ensure the survival of Treg cells.

Influencing the disease outcome by the history of infections

Previous studies have shown that prior infections with LCMV, MCMV, PV, BCG or influenza A virus can provide certain levels of heterologous protection during VACV infection, but the history of influenza infection impedes the clearance of LCMV and MCMV infections (Chen *et al.*, 2003; Mathurin *et al.*, 2009; Selin *et al.*, 1998). In Chapter VI, I showed that the history of LCMV infection had long-term effects on MCMV persistent infection. MCMV titers were higher in multiple organs in the LCMV-immune mice than in the BHK sham control mice. However, fewer inflationary memory

CD8 T cells were found at 12 weeks post infection. The higher viral titers and fewer inflationary memory T cells contradict the general understanding that higher viral load would enhance memory inflation. As memory inflation depends on the processing by the constitutive proteasomes and presentation by MHC class I molecules on non-hematopoietic cells (Hutchinson *et al.*, 2011; Seckert *et al.*, 2011; Torti *et al.*, 2011a), future studies will have to determine what remote effects LCMV might have, after the infection has long been cleared, on the proteasomes and/or the non-hematopoietic cells that affect only the establishment of the inflationary memory T cells but not of the non-inflationary memory T cells. One previous study suggests that the infected non-hematopoietic cells responsible for stimulating inflationary memory T cells reside in the lymph nodes, where MCMV does not persist (Torti *et al.*, 2011a). It may be possible that these cells become less susceptible to infection by MCMV in the LCMV-immune mice, and hence are less active in promoting memory inflation. Identifying the cell type responsible for memory inflation may be an important next step to solving this mystery. I found that the history of LCMV infection also caused more severe immunopathology in the fat pads early during MCMV infection. Enhanced or altered lung immunopathology has been demonstrated in VACV-infected LCMV-immune mice and LCMV- or MCMV-infected influenza-immune mice, and is found associated with the infiltration of CD8 T cells and macrophages (Chen *et al.*, 2001a; 2003). In preliminary analysis of the MCMV-infected mice, I found increased presence of macrophages, CD8 T cells and NK1.1+ cells in the fat pads of LCMV-immune mice, but their association with necrosis and tissue damage after MCMV infection is yet to be confirmed with more experiments.

My study with the LCMV-infected DC-immunized VACV-immune mice suggested a link between the number of cross-reactive CD8 T cells and the immunoprotection during heterologous infections. It has been shown that VACV clearance is more efficient in the LCMV-immune mice; however, VACV-immune mice were not protected during LCMV infection (Selin *et al.*, 1998). Cross-reactive CD8 T cells from LCMV-immune mice have been shown to mediate heterologous immunoprotection against VACV (Cornberg *et al.*, 2010). The CD8 T cell response is important in the control of LCMV infection. Therefore, we hypothesized that the difference in the CD8 T cell populations might explain the non-reciprocity of heterologous protection between LCMV and VACV. In Chapter VII, I showed that the memory CD8 T cells generated from LCMV and VACV (VACV-GP) infections were phenotypically different, but they both proliferated and produced granzyme B upon *in vitro* peptide stimulation. The memory phenotype analysis also suggested that the memory CD8 T cells from VACV-immune mice should be capable of rapid proliferative recall response. Since the number of known potentially cross-reactive CD8 T cells is ten times less in the VACV-immune mice, I tested whether increasing the cross-reactive memory CD8 T cells in the VACV-immune mice might protect the mice during LCMV infection. DC-immunization successfully increased the frequency of the cross-reactive A11R₁₉₈₋₂₀₅-specific CD8 T cells in VACV-immune mice, some of which were able to reduce the LCMV titers. However, the frequency of A11R₁₉₈₋₂₀₅-specific CD8 T cells in blood before LCMV challenge did not predict protection. The private specificity of T cell repertoire likely dictates the probability of recognizing a cross-reactive epitope. A

previous study has found a more stringent requirement on the peptide sequences recognized by the A11R₁₉₈₋₂₀₅-specific CD8 T cell lines generated from VACV-immune mice compared to those recognized by the GP₃₄₋₄₁-specific CD8 T cell lines generated from LCMV-immune mice, suggesting that the differences in the TCR repertoire may explain the reduced likelihood of the A11R₁₉₈₋₂₀₅-specific CD8 T cell lines cross-reacting with LCMV GP₃₄₋₄₁ peptide (Shen *et al.*, 2013). Although the possibility of the A11R₁₉₈₋₂₀₅-specific CD8 T cell lines responding to the other potentially cross-reactive LCMV epitopes were not shown in that study, it is still arguable that the memory CD8 T cells from VACV-immune mice may not be as cross-reactive to LCMV as the memory CD8 T cells from LCMV-immune mice are to VACV. Further analysis of the A11R₁₉₈₋₂₀₅-specific CD8 T cells from the DC-immunized VACV-immune mice after LCMV challenge found a correlation between protection and the net frequency of A11R₁₉₈₋₂₀₅-specific CD8 T cells after background subtraction at day 6 post LCMV infection. Mice that could clear LCMV infection more efficiently tended to have 0.59% or higher percentage of CD8 T cells being A11R₁₉₈₋₂₀₅-specific at day 6 post infection, and the cutoff was significant by Fisher's exact test. Therefore, both private specificity and the number of cross-reactive memory CD8 T cells determine the outcome of heterologous infection between LCMV and VACV.

I also found that a higher proportional response to NP₃₉₆₋₄₀₄ after LCMV challenge might be more protective. The analysis of the CD8 T cell specificity in VACV-immune mice at day 6 post LCMV infection suggested a correlation between the proportional responses and immunoprotection. DC-immunized VACV-immune mice

that were protected during LCMV infection had a significantly higher proportional response to NP₃₉₆₋₄₀₄ and lower proportional responses to NP₂₀₅₋₂₁₂ and GP₁₁₈₋₁₂₅. This is consistent with the observation that NP₃₉₆₋₄₀₄-specific T cell lines are more sensitive to cognate antigen and are more efficient in eliminating infected targets (Gallimore *et al.*, 1998), and may explain why some mice with cross-reacted A11R₁₉₈₋₂₀₅-specific memory CD8 T cells were not protected during LCMV infection because cross-reactivity that results in a lower proportional response to NP₃₉₆₋₄₀₄ may not be as effective in controlling LCMV infection. Although the GP₃₄₋₄₁-specific memory CD8 T cells are the second most cross-reactive to VACV during acute infection (Kim *et al.*, 2005), no difference in the GP₃₄₋₄₁-specific proportional responses at day 6 post LCMV infection was found between the VACV-immune and the DC-immunized VACV-immune groups regardless of protection status during LCMV infection. It is possible that the cross-reactivity of VACV-specific memory CD8 T cells towards GP₃₄₋₄₁ may be weak and hence infrequently affects the proportional response during LCMV infection.

In conclusion, I have shown in this thesis that the immunoproteasomes were important in shaping LCMV-specific CD8 T cell response and memory. I confirmed that the number and frequency of memory CD8 T cells could be reduced by heterologous viral infections, and showed that memory CD4 T cells might suffer the same fate; however, persistent MCMV infection did not cause continuous erosion of pre-existing memory CD8 or CD4 T cells, suggesting that memory T cell loss was an active process mainly due to IFN $\alpha\beta$ -associated early events. I also found that Treg cells remained

relatively stable in numbers and were resistant to apoptosis during LCMV infection. The relative stability of Treg cells may explain why infections do not lead to high incidence of autoimmunity. I found that the history of LCMV infection had detrimental effects on MCMV infection, and the low number of potentially cross-reactive CD8 T cells in VACV-immune mice might have played a part in the non-reciprocity of immunoprotection between LCMV and VACV. These findings illustrate the dynamic interaction between T cell immunity and viral infections. With the multiple aspects involved, a wide spectrum of responses to an infection is possible in a population with diverse genetic backgrounds and histories of infections. Moreover, the immune system is continuously modulated by infections, changing the response to subsequent challenges.

Appendix – Peptide sequences used in this thesis

Table 1. MCMV-specific MHC I-restricted peptides (Munks *et al.*, 2006a, b).

MCMV K ^b -restricted		MCMV D ^b -restricted	
m139 ₄₁₉₋₄₂₆	TVYGFCLL	M45 ₉₈₅₋₉₉₃	HGIRNASFI
M57 ₈₁₆₋₈₂₄	SCLEFWQRV	M86 ₁₀₆₂₋₁₀₇₀	SQNINTVEM
m141 ₁₆₋₂₃	VIDAFSRL	M33 ₄₇₋₅₅	GGPMNFVVL
M38 ₃₁₆₋₃₂₃	SSPPMFRV	M112 ₁₇₁₋₁₇₉	AAVQSATSM
M78 ₈₋₁₅	VDYSYPEV	M44 ₁₃₀₋₁₃₈	ACVHNQDII
M100 ₇₂₋₇₉	RIIDFDNM	m164 ₂₆₇₋₂₇₅	WAVNNQAIV
m164 ₂₈₃₋₂₉₀	GTTDFLWM	M77 ₄₇₄₋₄₈₂	GCVKNFEFM
M97 ₂₁₀₋₂₁₇	IISFPFGL	m04 ₁₋₉	MSLVCRLVL
M38 ₃₈₋₄₅	STYTFVRT	M36 ₂₁₃₋₂₂₁	GTVINLTSV
M102 ₄₄₆₋₄₅₅	SIVDLRFAVL		
M31 ₂₉₇₋₃₀₅	VAPDFGVRM		
IE3 ₄₁₆₋₄₂₃	RALEYKNL		
* M57 ₇₂₇₋₇₃₄	KSVEFERI		

* Novel cross-reactive epitope identified in this work.

Table 2. LCMV-specific MHC I-restricted peptides (Kotturi *et al.*, 2007).

LCMV K ^b -restricted		LCMV D ^b -restricted	
GP ₃₄₋₄₁	AVYNFATC	GP ₃₃₋₄₁	KAVYNFATC
L ₃₄₉₋₃₅₇	SSLIKQSKF	NP ₃₉₆₋₄₀₄	FQPQNGQFI
GP ₁₁₈₋₁₂₅	ISHNFCNL	GP ₂₇₆₋₂₈₆	SGVENPGGYCL
NP ₂₀₅₋₂₁₂	YTVKYPNL	L ₄₅₅₋₄₆₃	FMKIGAHPI
L ₂₀₆₂₋₂₀₆₉	RSIDFERV	L ₆₈₉₋₆₉₇	KFMLNVSYL
NP ₂₃₈₋₂₄₈	SGYNFSLGAAV	L ₃₃₈₋₃₄₆	RQLLNLDVL
L ₁₅₆₋₁₆₃	ANFKFRDL	GP ₉₂₋₁₀₁	CSANNSHHYI
L ₁₈₇₈₋₁₈₈₅	GPFQSFSV	NP ₁₆₆₋₁₇₅	SLLNNQFGTM
L ₁₄₂₈₋₁₄₃₅	NSIQRRTL		

Table 3. LCMV-specific MHC II-restricted peptides (Homann *et al.*, 2001).

GP ₆₁₋₈₀	GLNGPDIYKGVYQFKSVEFD
NP ₃₀₉₋₃₂₈	SGEGWPYIACRTSVVGRAWE

Table 4. VACV-specific MHC I-restricted peptides (Kim *et al.*, 2005; Moutaftsi *et al.*, 2006).VACV K^b-restricted

B8R ₂₀₋₂₇	TSYKFESV
A47L ₁₃₈₋₁₄₆	AAFEFINSL
E7R ₁₃₀₋₁₃₇	STLNFNNL
A11R ₁₉₈₋₂₀₅	AIVNYANL

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