Role of TNF in Heterologous Immunity between Lymphocytic Choriomeningitis Virus and Vaccinia Virus: A Dissertation

Siwei Nie
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The Role of TNF in Heterologous Immunity between
Lymphocytic Choriomeningitis Virus and
Vaccinia Virus

A Dissertation Presented
By

Siwei Nie

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

October, 2008

Immunology and Virology
The Role of TNF in Heterologous Immunity between Lymphocytic Choriomeningitis Virus and Vaccinia Virus

A Dissertation Presented
By
Siwei Nie

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Program in Immunology and Virology
November 14th, 2008
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I would also like to thank my parents and my brother for their continuous and limitless love and support, which I have always depended on. Knowing that they would be proud of the progress I have made is the strongest motivation behind my success. Lastly, I would like to thank my husband Quan Yuan for always being with me.
ABSTRACT

Prior immunity to a related or unrelated pathogen greatly influences the host’s immune response to a subsequent infection and can cause a dramatic difference in disease course, a phenomenon known as heterologous immunity. Heterologous immunity can influence protective immunity, immunopathology and/or immune deviation of cytokine-producing T cell subsets. Examples of heterologous immunity have been well documented in mouse models, as well as during human infections. For example, prior immunity to lymphocytic choriomeningitis virus (LCMV) provides partial protection against vaccinia virus (VV), as LCMV-immune mice show reduced VV titers and increased survival upon lethal dose VV infection. Heterologous protection against VV challenge, as a result of LCMV immunity, is mediated by LCMV-specific CD4 and CD8 T cells, as transfer of LCMV-specific memory T cells can mediate this protective effect in naïve mice. The recognition of a single TCR with more than one MHC-peptide complex is referred to as T cell cross-reactivity. A VV K\textsuperscript{b}-restricted epitope a11r\textsubscript{198} was identified to be able to induce cross-reactive responses from LCMV-specific CD8 T cells. During VV infection, LCMV-specific memory T cells that are cross-reactive to VV epitopes produce IFN-\textgreek{g} early in VV infection. IFN-\textgreek{g} is essential for mediating the protection against VV in LCMV-immune mice, as this heterologous protection is absent in IFN-\textgreek{g}\textsuperscript{-/-} and IFN-\textgreek{g} blocking antibody-treated LCMV-immune mice. In addition to
protective immunity, cross-reactive LCMV-specific memory T cells and IFN-\(\gamma\) also induce an altered immunopathology during heterologous VV challenge. LCMV-immune mice show moderate to severe levels of inflammation of the fat tissue, known as panniculitis, in the visceral fat pads upon VV challenge. In humans, panniculitis is a painful condition, most commonly presenting as erythema nodosum. Erythema nodosum is a disease of unknown etiology with no known treatment. It may occur following intracellular bacterial and viral infections, and occasionally happens after vaccination with VV for smallpox. During infections there can be a delicate balance between the ability of immune responses to provide protective immunity, and the tendency to induce immunopathology. By using the mouse model of heterologous immunity between LCMV and VV, we tried to understand how the immunity to LCMV biased the balance between the protective immunity and immunopathology, and what effector molecules were responsible for the pathogenesis of panniculitis in this system.

TNF is a pleiotropic cytokine, which is required for normal innate and adaptive immune responses. Its functions range from inducing proliferative responses including cell survival, to destructive responses such as promoting apoptosis and programmed necrosis. In response to inflammatory stimuli, activated macrophages/monocytes produce large amounts of TNF, and upon activation, T cells, B cells and NK cells also produce TNF. In vitro and in vivo studies have shown that TNF in synergy with IFN-\(\gamma\) plays an important role in mediating host
defense against pathogens, such as *Listeria monocytogenes* and poxviruses in mice and hepatitis B virus and human immunodeficiency virus in humans. However, inappropriate expression of TNF often results in tissue damage. Considering the important role TNF plays in both host defense and mediating autoimmune diseases, we hypothesized that TNF was required for mediating both protective and pathogenic effects in the heterologous immunity between LCMV and VV.

We first examined whether TNF was involved in mediating protective heterologous immunity. LCMV-immune mice, that were TNF-deficient as a consequence of genetic deletion (TNF−/−) or receptor blockade by treatment with etanercept (TNFR2: Fc fusion protein), were challenged with VV. These TNF-deficient mice showed normal recruitment and selective expansion of cross-reactive LCMV-specific memory CD8 T cells. They also exhibited efficient clearance of VV similar to LCMV-immune mice with normal TNF function. Thus, we concluded that neither TNF nor lymphotoxin (LT), which uses the same receptors as TNF, was required in mediating protective heterologous immunity against VV. Indeed, prior immunity to LCMV could completely compensate for the role of TNF in protection of naïve mice against VV infection, even under conditions of lethal dose inoculum. Thus, heterologous immunity may help explain why treatment of humans with etanercept is reasonably well tolerated with relatively few infectious complications.

One of the histological characteristics of panniculitis is necrosis of adipose
tissue. It is known that three members in the TNF superfamily, i.e. TNF/LT, FasL and TRAIL are able to induce necrosis of a target cell. It is also known that TNF is able to induce VV-infected cells to go through necrosis, when apoptosis is blocked in these cells by VV protein. Furthermore, TNF and FasL have already been shown to be associated with some skin and fat pathology. Thus, we hypothesized that TNF, FasL and TRAIL were involved in the pathogenesis of panniculitis in VV infected LCMV-immune mice. By using blocking antibodies or genetically deficient mice, we demonstrated that both TNF/LT and FasL were crucial for inducing panniculitis. Although TNFR1 has been reported to induce programmed necrosis, our data indicated that TNFR2, not TNFR1, was involved in mediating tissue damage in the fat pads of LCMV-immune mice infected with VV. We also found that TNF signaled through TNFR2 to up-regulate the expression of Fas on adipocytes. Thus, the engagement of Fas on the adipocytes with FasL expressed on activated VV-specific and cross-reactive LCMV-specific CD8 T cells in the fat pads could lead to panniculitis. Thus, our data may identify a potential mechanism in the pathogenesis of human panniculitis, and may suggest a possible treatment for this painful disease.

Recent reports suggest that heterologous immunity may contribute to the tremendous variation in symptoms between individuals, from subclinical to death, upon viral infection. Even in genetically identical mice, variations in immunopathology from none to life-threatening levels of pathology are observed in LCMV-immune mice during VV infection. By adoptive transfer of splenocytes from
a single LCMV-immune donor into two recipients, we showed that similar levels of pathology were generated in mice receiving the same splenocytes. However, the level of pathology varied among recipients receiving splenocytes from different LCMV-immune donors. The difference in levels of VV-induced pathology observed in individual LCMV-immune mice was a reflection of the private specificity of the T cell repertoire, which is a unique characteristic of each individual immune host.

The goal of this doctoral thesis is to understand how heterologous immunity contributes to the pathogenesis of panniculitis. Our data demonstrate that TNF/LT and FasL directly contribute to development of panniculitis in LCMV-immune mice during VV infection, and suggest that anti-TNF treatment might be a useful treatment for diseases, such as erythema nodosum and lupus-induced acute fatty necrosis in humans.
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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluoroscein succinimidyl ester</td>
</tr>
<tr>
<td>CT</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue haemorrhagic fever</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>death receptor</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EN</td>
<td>erythema nodosum</td>
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<tr>
<td>EV</td>
<td>ectromelia virus</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
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<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
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<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
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<tr>
<td>IAV</td>
<td>influenza A virus</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescent</td>
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<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
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<td>IFN-γR</td>
<td>interferon gamma receptor</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IM</td>
<td>infectious mononucleosis</td>
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<td>IN</td>
<td>intranasal</td>
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<td>IP</td>
<td>intraperitoneal</td>
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<tr>
<td>IV</td>
<td>intravenous</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LT</td>
<td>lymphotoxin</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescent intensity</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>NP</td>
<td>nucleoprotein</td>
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<td>Abbreviation</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PEC</td>
<td>peritoneal exudate cells</td>
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<tr>
<td>PFU</td>
<td>plaque forming unit</td>
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<tr>
<td>PP</td>
<td>Peyer's patches</td>
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<tr>
<td>PV</td>
<td>pichinde virus</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumor necrosis factor related apoptosis inducing ligand</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VV</td>
<td>vaccinia virus</td>
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<tr>
<td>WB</td>
<td>western blot</td>
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<tr>
<td>WR</td>
<td>western reserve</td>
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<td>WT</td>
<td>wild type</td>
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CHAPTER I: INTRODUCTION

CD8 T cells are a central component of host defense against intracellular pathogens. Following the resolution of the infection, a small proportion of the pathogen-specific CD8 T cells survive into memory T cells and provide long-term protection against re-infection with the same pathogen. Studies over the past decade suggest that memory T cells that are specific to antigens derived from previously encountered pathogens can cross-react with antigens from related or unrelated pathogens and alter the outcome of subsequent infections, a phenomenon known as heterologous immunity. Many examples of heterologous immunity have been reported in both murine and human systems (Welsh and Fujinami, 2007). For example, T cells that are specific to influenza A virus (IAV) epitopes can be cross-reactive to the epitopes from hepatitis C virus (HCV) and Epstein-Barr virus (EBV) (Clute et al., 2005; Urbani et al., 2005; Wedemeyer et al., 2001). The cross-reactivity between IAV and HCV has been suggested to be associated with more severe symptoms in patients with acute HCV infection (Urbani et al., 2005). In mice, prior immunity to lymphocytic choriomeningitis virus (LCMV) can provide partial protection against subsequent infection with Pichinde virus (PV) or vaccinia virus (VV) (Selin et al., 1998).

Our group has extensively studied heterologous immunity between LCMV and VV, and has demonstrated that both LCMV-specific memory CD8 and CD4 T cells are required to mediate partial protection against VV in LCMV-immune mice.
(Selin et al., 1998). This was demonstrated by adoptive transfer of CD4 or CD8 depleted splenocytes from LCMV-immune donors into naïve mice and challenging with VV (Selin et al., 1998). In addition to heterologous protection, LCMV-specific memory T cells mediate altered immunopathology during VV infection, as LCMV-immune mice develop panniculitis, in the form of acute fatty necrosis, in the visceral fat pads upon VV infection (Selin et al., 1998; Yang et al., 1985). The ability of LCMV-specific memory T cells to mediate protection and induce immunopathology during VV infection is dependent on IFN-γ. In the absence of IFN-γ either by blocking anti-IFN-γ antibodies or using IFN-γ receptor knockout (KO) mice, the protective and pathogenic effects of LCMV-specific memory T cells are completely abrogated (Selin et al., 1998). The mechanisms for the pathogenesis of panniculitis in this system are still not completely understood, but our data shown in this thesis indicate that TNF may also be involved.

TNF is an important cytokine that regulates immune responses at many levels. TNF is produced by macrophages/monocytes, T cells, B cells and NK cells upon activation (Aggarwal, 2003), and modulates both innate and adaptive immune responses (Vassalli, 1992). As shown by in vitro and in vivo studies in both mice and humans, TNF plays important roles in host defense against bacterial and viral infections (Schluter and Deckert, 2000). At the same time, TNF is also implicated in tissue damage and autoimmune disease. TNF is reported to be involved in the pathogenesis of rheumatoid arthritis (RA), Crohn’s disease, chronic heart failure
as well as erythema nodosum (EN), the most common form of panniculitis in humans (Aggarwal, 2003; Requena and Yus, 2008).

The main interest of this thesis research is to investigate the role of TNF in mediating heterologous protective immunity and immunopathology in LCMV-immune mice challenged with VV. We hypothesized that TNF, like IFN-γ, would be required for both protective and pathogenic effects. Our initial approach to test the hypothesis was to block the effects of TNF by using anti-TNF treatment (etanercept, human TNFR2: Fc fusion protein) or TNF−/− mice. To determine if TNF was required to mediate heterologous protective immunity against VV in LCMV-immune mice, we compared VV levels between naïve and LCMV-immune mice in the presence or absence of TNF after VV challenge. We also compared the severities of panniculitis in LCMV-immune mice with or without TNF to reveal the influence of TNF on inducing panniculitis.

A. T cell receptor repertoire and cross-reactive T cells

To give the host efficient protection against a wide array of pathogens, T cell receptor (TCR) repertoire requires an extensive diversity. Through the processes of TCR gene rearrangement, editing at junctions and pairing TCR-α and β segments, there is potential for more than 10^{15} TCR generated (Fig 1.1). After positive and negative selection in the thymus, the diversity of TCR is reduced
**Figure 1.1 Structural diversity of TCR.** TCR is generated by DNA segment recombination. Additional structural diversity is mediated by addition and deletion of nucleotides at the junctions between segments. The generated TCR then goes through positive and negative selection in the thymus. This graph was adapted from Nikolich-Zugich J 2004 Nat Rev Immunol 4: 123-132.

Combinatorial diversity: $5.2 \times 10^{15}$ possible TCR-αβ

Thymic selection: more than $1 \times 10^{13}$ possible TCRs could be selected

Experimental detection in the periphery: $2 \times 10^6$ in mice and $2 \times 10^7$ in humans
3-100 fold. Therefore, the theoretical diversity of TCR is more than $1 \times 10^{13}$. However, the estimates of the diversity of TCR in the periphery of mouse and human are $10^6$-$10^7$ and $10^7$-$10^8$ mature T lymphocytes with different sequences in each individual at a given time, respectively (Arstila et al., 1999; Casrouge et al., 2000; Nikolich-Zugich et al., 2004).

Naïve T cells are activated after contact with peptides presented by major histocompatibility complex (MHC) molecules in combination with co-stimulatory signals. Activated T cells go through programmed proliferation and become effector T cells. After antigen clearance, most of these effector cells undergo apoptosis, while a small portion survive and become memory T cells. Compared to naïve T cells, memory T cells have a number of advantages to provide rapid protection against a re-encountered pathogen. First, they are present at a higher frequency than naïve precursors. Second, they have a lower activation threshold and can respond to antigens at a lower concentration (Pihlgren et al., 1996). Third, they are less dependent on co-stimulatory signals (Dutton et al., 1998; Sprent and Surh, 2002; Welsh et al., 2004).

Since T cell recognition of the peptide-MHC complex relies on only a few, but not all amino acid residues of the peptide, it is possible that one T cell can recognize more than one peptide bound to self MHC molecules, which is defined as T cell cross-reactivity (the cross-reactivity on non-self MHC molecules is not discussed in this thesis). Mathematical calculation suggests that a single TCR has the potential to recognize about $10^6$ different peptide-MHC combinations (Mason,
In fact, cross-reactivity is observed between peptides with similar sequences, with minimal similarity and with unrelated sequences (Wilson et al., 2004). Two possible mechanisms of cross-reactivity are shown in figure 1.2. Molecular mimicry is a mechanism whereby a T cell uses the same receptor elements to interact with different peptides with large similarities in sequence and structure. On the other hand, a TCR is able to cross-react with a peptide with little or no sequence or structural similarity as a result of the conformational adjustment of the receptor and usage of different determinants of the TCR and peptides, referred as alternative recognition (Boniface et al., 1999; Daniel et al., 1998; Speir et al., 1998).

A number of studies have shown evidence of cross-reactive T cell responses during human and murine viral infections. T cell cross-reactivity can involve peptides derived from the same viral protein (Anderson et al., 1992; Kulkarni et al., 1993), different proteins encoded by the same virus (Anderson et al., 1992; Kuwano et al., 1991), similar proteins of different serotypes or related viruses (Brehm et al., 2002; Mongkolsapaya et al., 2003), and different proteins from unrelated viruses (Acierno et al., 2003; Clute et al., 2005; Kim et al., 2005; Nilges et al., 2003; Urbani et al., 2005). In the presence of cross-reactive immune responses, the consequence of a heterologous virus infection can be altered, a phenomenon termed as heterologous immunity. For example, prior immunity to LCMV can greatly influence the consequences of subsequent VV infection, resulting in rapid clearance of VV, as well as inducing immunopathology.
Figure 1.2 Potential mechanisms of cross-reactivity of TCR with different peptides. The left panel shows the interaction of a TCR with a peptide presented by a MHC molecule. The middle panel shows an alternative peptide that has similar determinants and interacts with the same TCR in the same manner as the first peptide, referred to as molecular mimicry. The right panel shows a situation in which different determinants of the TCR interact with the presented peptide, referred to as alternative recognition. This model was adapted from Welsh and Selin 2002 Nat Rev Immunol 2: 417-426.
Cross-reactive CD8 T cell epitopes between LCMV and VV have been identified, and these cross-reactive LCMV-specific memory T cells and IFN-γ have been shown to be important in mediating heterologous immunity (Chen et al., 2001; Kim et al., 2005; Selin et al., 1998). Thus, we were able to utilize this heterologous immunity system to test our hypothesis that TNF was required for mediating heterologous protective immunity and immunopathology in LCMV-immune mice upon VV challenge.

B. Heterologous immunity between LCMV and VV

Many murine models have suggested that the effect of heterologous immunity on subsequent infections includes partial protective immunity, altered immunopathology and immune deviation (Welsh and Selin, 2002). One example of protective immunity is described in mice sequentially infected with LCMV and pichinde virus (PV). Three to four days after heterologous challenge, immune mice show a 2-200-fold reduction in viral titers, compared to immunologically naïve mice (Selin et al., 1998). However, the protective heterologous immunity is not reciprocal, as protection against PV provided by LCMV immunity is better than protection against LCMV provided by PV immunity (Selin et al., 1998). Similarly, LCMV-immune mice show reduced VV titers and increased survival upon lethal dose VV infection (Chen et al., 2001), while VV-immune mice are not resistant to
LCMV challenge (Selin et al., 1998). This lack of reciprocity may be explained by VV having greater potential to activate cross-reactive LCMV-specific memory T cells, since it is such a large virus and encodes more than 200 proteins and thousands of potential epitopes (Goebel et al., 1990). As demonstrated previously, protection against VV challenge requires both LCMV-specific CD4 and CD8 memory T cells (Selin et al., 1998). Adoptive transfer of LCMV-specific CD4 and CD8 memory T cells together can mediate this protective effect in naïve mice during VV challenge, while deletion of either population prior to transfer leads to the loss of protection. LCMV-specific CD8 memory T cells are recruited to and accumulate in the infected peripheral tissue during VV challenge, and cross-reactive LCMV-specific T cells are stimulated to produce IFN-γ as early as day 3 post VV infection (Chen et al., 2001). By screening VV peptides with sequence similarity to the LCMV epitope NP\textsubscript{205}, two VV epitopes, e7r\textsubscript{130} and a11r\textsubscript{198} were identified by Cornberg \textit{et. al.} (Cornberg et al., 2007). Both e7r\textsubscript{130} and a11r\textsubscript{198} have at least 30% sequence similarity to LCMV-NP\textsubscript{205}. However, only a11r\textsubscript{198} induced cross-reactive CD8 T cell responses from LCMV memory CD8 T cells that were specific for GP\textsubscript{118}, GP\textsubscript{34} and NP\textsubscript{205} (Fig 1.3; Cornberg et al., manuscript submitted). The selective activation and expansion of these cross-reactive LCMV-specific memory CD8 T cells upon VV challenge led to a change in the hierarchies of the LCMV-specific responses. The cross-reactive responses elicited in different mice was distinct, and thus the skewing of LCMV
Figure 1.3 Network of T cell cross-reactivity within murine viral infections. VV epitope a11r can induce cross-reactive CD8 T cells specific to epitopes derived from other VV proteins as well as epitopes derived from other unrelated viruses, such as LCMV and PV. This figure was taken from Cornberg unpublished data.
responses also varied between individuals (Kim et al., 2005).

Cross-reactivity between LCMV and VV not only provides protection against VV, but also alters the disease pathogenesis associated with VV infection. Naïve mice that are infected intraperitoneally (IP) with VV have little pathology in the visceral fat pad, while LCMV-immune mice show moderate to severe levels of inflammation of the fat tissue (panniculitis) after VV infection (Selin et al., 1998). During VV intranasal (IN) infection, naïve mice show mainly severe pulmonary edema and neutrophilic infiltrate in the lung, whereas LCMV-immune mice have little edema but increased mononuclear infiltration and bronchiolitis obliterans (Chen et al., 2001). In humans, panniculitis and bronchiolitis obliterans are diseases of unknown etiology and sometimes occur after viral and intracellular bacterial infections. Similar to the protective immunity against VV, the immunopathology is also mediated by LCMV-specific memory T cells, as transfer of splenocytes from LCMV-immune mice into naïve mice could induce severe panniculitis after VV infection. In this condition, deletion of both CD4 and CD8 cells was required prior to transfer to abrogate this effect, suggesting that either population could mediate the pathogenesis of panniculitis (Selin LK, unpublished data).

Figure 1.4 summarizes the mechanism of heterologous immunity in LCMV-immune mice during VV challenge. VV epitopes presented by antigen presenting cells (APC) activate the cross-reactive LCMV-specific T cells, which
Figure 1.4 Model of heterologous immunity in LCMV-immune mice during VV infection. Some of the LCMV-specific memory T cells are cross-reactively stimulated by antigens from VV. This causes the release of IFN-γ, which further activates APCs and enhances their expression of MHC molecules. Together, these events antagonize viral replication and, at the same time, facilitate the development of immunopathological lesions. This model was adapted from Welsh and Selin 2002 Nat Rev Immunol 2: 417-426.
produce large amounts of IFN-\(\gamma\). Studies using anti-IFN-\(\gamma\) treatment and IFN-\(\gamma\)R\(^{-/}\) mice, have shown that IFN-\(\gamma\) plays a crucial role in mediating protective immunity against VV and in inducing immunopathology in LCMV-immune mice (Selin et al., 1998). The protective immunity against VV is missing in IFN-\(\gamma\)R\(^{-/}\) LCMV-immune mice, and the levels of panniculitis are diminished in anti-IFN-\(\gamma\) treated or IFN-\(\gamma\)R\(^{-/}\) LCMV-immune mice.

C. The effect of private specificity on heterologous immunity

The hierarchies of epitope-specific T cell responses to a given pathogen are often similar in genetically identical mice. One example is that LCMV infection of naïve C57BL/6 mice generates a CD8 T cell response to LCMV epitopes in a common hierarchy, as the response is co-dominated by NP\(_{396}\) and GP\(_{33/34}\) responses, while GP\(_{276}\), NP\(_{205}\) and GP\(_{118}\) responses are subdominant (Kim et al., 2005). Similar hierarchies of LCMV-specific responses are used in most of LCMV-infected naïve C57BL/6J mice. This is a form of public specificity in TCR repertoire. However, at the clonal level the T cell response against a given epitope differs between individuals, as TCR with different CDR3 amino acid sequences are used in the epitope-specific response by different individuals, these leading to private specificity of TCR repertoire (Welsh, 2006). The most comprehensive explanation for this individual variation in TCR usage by an epitope-specific
response is the difference in the peripheral T cell pool generated in each host. The independent and random rearrangement of DNA segments generates a TCR repertoire with wide variety in an individual. The potential diversity of peripheral TCR repertoire is $\sim 10^{13}$ for mice and $\sim 10^{16}$ for human, and the total number of unique T-cell clonotypes is $\sim 10^6$ for mice and $\sim 10^7$ for humans (Nikolich-Zugich et al., 2004; Venturi et al., 2008). Differences in expressed TCR repertoires are expected, even between genetically identical animals. In fact, an analysis of naïve T cells in individual mice of the same inbred strain shows that the naïve repertoire is mostly non-overlapping in distinct animals (Bousso et al., 1998). Random stochastic encounters of T cells with antigen also cause differences in TCR usage for a specific response. After transfer of naïve T cells derived from a single donor, two recipients generate different TCR repertoires in response to the same peptide stimulation (Attuil et al., 2000). Thus, differences in the peripheral naïve TCR repertoires and the random process of antigen-T cell engagement both contribute to private specificity in antigen-specific TCR repertoires (Welsh, 2006).

After heterologous challenge, the frequency of memory T cells that are specific for previously encountered antigen is significantly decreased (Selin et al., 1999). The mechanisms for the decreased frequency of memory T cells include dilution by the accumulation of new effector cells, and the apoptotic death of about one third of the pre-existing memory T cells that are not cross-reactive to the heterologous pathogen. However, after a transient attrition, a small subset of memory T cells that cross-react with the current infecting pathogen expand and
give rise to a skewed hierarchy in the T cell response specific to the original antigen. In PV challenged LCMV-immune mice, the T cell response against LCMV-NP$_{205}$, which is cross-reactive to PV-NP$_{205}$, shows a dramatic increase, while the normally dominant LCMV-NP$_{396}$ and GP$_{33}$ responses are remarkably decreased (Brehm et al., 2002). Although an enhancement of NP$_{205}$ responses was shown in all LCMV-immune mice challenged with PV, individual variations in magnitude as well as TCR usage were found (Cornberg et al., 2006). This variation is a reflection of private specificity of the memory T cell repertoire. Another example of private specificity is the unpredictable expansion of LCMV-specific CD8 T cells in LCMV-immune mice infected with VV (Chen et al., 2001; Kim et al., 2002; Kim et al., 2005). As shown in Figure 1.3, cross-reactivity between LCMV and VV is more complex than cross-reactivity between LCMV and PV. The proliferation of cross-reactive T cells in response to VV challenge is not always the same in different mice. Indeed, 50% of VV-infected LCMV-immune mice showed selective expansion of LCMV-NP$_{205}$ responses, and 15% and 12% of mice showed an increase in GP$_{33/34}$- and GP$_{118}$-specific responses, respectively (Kim et al., 2005). To understand the cause of this variation, a modified adoptive transfer technique was used (Fig 1.5). Naïve mice receiving splenocytes derived from a single LCMV-immune donor exhibited similar expansion of LCMV-specific responses, while mice receiving different donor cells exhibited unique responses (Kim et al., 2005). Thus, differences in the VV-induced
Figure 1.5 Experimental design used for studying the role of private specificity in the pattern of cross-reactivity. CFSE-labeled LCMV immune donor cells derived from a single donor were transferred into multiple hosts, which were infected with VV. This graph was adapted from Kim SK 2005 J Exp Med 201: 523-533.
patterns of cross-reactivity among LCMV-immune mice are determined by the private specificity of the memory T cells within the individual host.

In a phenomenon similar to the variations in cross-reactive responses induced in LCMV-immune mice by VV infection, the levels of panniculitis generated in these mice are also different between individuals. The severity of panniculitis varies from none to life-threatening levels. Panniculitis in VV-infected LCMV-immune mice is mediated by cross-reactive LCMV-specific memory T cells. Therefore, we hypothesized that the variations in levels of panniculitis were also influenced by private specificity.

D. Panniculitis: inflammation of fat tissue

Panniculitis is a group of diseases that involve inflammation of adipose tissues (Requena and Yus, 2001). It presents as single or multiple crops of tender nodules in fat tissue. Histological analysis shows fat cell necrosis, infiltration of inflammatory cells and fat-filled macrophages in acute panniculitis. Panniculitis may occur in both cutaneous and visceral fat tissue, and may result from a variety of conditions. Usually, panniculitis without systemic disease is due to trauma or cold. Panniculitis with systemic disease includes lupus, scleroderma, lymphomas and pancreatitis (Requena and Yus, 2001). There are several forms of cutaneous panniculitis, including erythema nodosum (EN) and erythema nodosum leprosum.
Panniculitis in the form of acute fatty necrosis occurs in LCMV-immune mice after VV infection. It is histologically characterized by the infiltration of inflammatory cells and necrosis of fat cells (Fig 1.6) (Selin et al., 1998; Yang et al., 1985), which is quite similar to human EN. EN is the most common form of human panniculitis (Requena and Yus, 2008). It presents with painful, tender, erythematous nodules usually 1-5 cm in diameter, and is most commonly located on the anterior portion of the lower legs, arms and soles. The lesions may be associated with fever, malaise, and joint pain. The etiology of EN is unknown, but it is mostly associated with fungal diseases, infectious mononucleosis, intracellular bacterial and viral infections and inflammatory bowel disease. It has also been reported that EN may occur after vaccination, including with VV which is used to vaccinate against smallpox (Gaertner et al., 2004). When the underlying disease is identified, patients usually respond well to the treatment. However, in a significant portion of EN, an association can not be found (Requena and Yus, 2008). In such situations, anti-inflammatory drugs and cortisone are used as standard treatment. But it is common that EN patients fail to respond to this treatment.

In the mid 1960s, thalidomide had been reported to have dramatic efficacy in treatment of erythema nodosum leprosum, another pathologic variant of panniculitis (Deng et al., 2003). Although the mechanism of action for thalidomide is still not completely clear, it has been reported that thalidomide selectively
Figure 1.6 Comparison of pathology in VV-infected LCMV-immune mice, and in human diseases. A, comparison of visceral fat showing panniculitis isolated from LCMV-immune mice challenged with VV, to normal-looking fat from naïve mice acutely infected with VV and uninfected LCMV-immune mice. B shows the histology of visceral fat with panniculitis from a VV-infected LCMV-immune mouse. C, histological features of human EN. This picture was taken from Welsh and Selin 2002 Nat Rev Immunol 2:417-426.
inhibits the production of TNF in human peripheral monocytes (Sampaio et al., 1991) by accelerating the degradation of TNF mRNA (Moreira et al., 1993). Another study done in mice suggests that Thalidomide reduces TNF production and antigen presentation by Langerhans cells (Deng et al., 2003). Recently, Boyd reported that a patient suffering from EN with unknown association had no response to standard therapeutic reagents, but significantly improved after receiving anti-TNF therapy (Boyd, 2007). Thus, these data suggest that TNF may play a role in EN.

In this thesis, we use the mouse model of heterologous immunity between LCMV and VV to test if TNF is involved in mediating panniculitis in LCMV-immune mice infected with VV.

E. “Cross-talk” between adipose tissue and immune system

Historically, fat cells were believed to be just containers for energy, which was released when the body required it. This paradigm was challenged when Hotamisligil first demonstrated that fat cells could produce TNF, a very important cytokine for both normal immune responses and autoimmune diseases (Hotamisligil et al., 1993). Production of TNF by fat tissue is responsible for the induction of insulin resistance, as neutralization of TNF can increase sensitivity to insulin in obese-diabetic animals (Hotamisligil et al., 1993). This study suggested
that adipose tissue has significant biological functions.

Fat cells can produce many different cytokines and adipokines, in addition to TNF. These cytokines and adipokines are involved in the induction of fat tissue inflammation, type 2 diabetes and cardiovascular disease (Powell, 2007). It has been suggested that TNF produced locally within adipose tissue is sequestered by the large amount of TNF receptors (TNFR) expressed in the tissue (Mohamed-Ali et al., 1999). However, TNF can induce adipocytes to express other cytokines, including IL-6 and leptin, through which the fat tissue can “cross-talk” to the immune system. Leptin, one of the most studied cytokines in adipocytes, plays a regulatory role in both innate and adaptive immunity (Matarese et al., 2007). In innate immunity, leptin elevates the activation of macrophages and increases the production of pro-inflammatory cytokines, such as TNF, IL-6 and IL-12. For adaptive immunity, leptin is important for thymic homeostasis during ontogeny, and for lymphocyte activation in the periphery. Furthermore, leptin can also polarize Th1 cytokine production and negatively regulate the proliferation of regulatory T cells, which may explain the association of leptin with some autoimmune diseases (Matarese et al., 2007).

Fat cells are also responding to cytokines, including TNF. TNF regulates the expression of some adipose-specific genes, including GLUT4 and free fatty acid, which are important for inducing insulin resistance (Ruan et al., 2002). It also modulates the metabolism of adipocytes, such as mediating cachexia, a wasting syndrome (Beutler and Cerami, 1986). As fat cells increase in size, TNF
expression is correspondingly increased, which in turn limits the growth of these cells. TNF negatively regulates adipose tissue mass by blocking the differentiation of pre-adipocytes, de-differentiating mature adipocytes and inducing apoptosis of adipocytes and pre-adipocytes (Coppack, 2001). Visceral pre-adipocytes are more sensitive to TNF-induced apoptosis than subcutaneous pre-adipocytes (Niesler et al., 1998), and the apoptosis of adipocytes induced by TNF is believed to be transduced by TNF receptor (TNFR) 1 (Hube and Hauner, 1999).

In this thesis, we are interested in the effect of TNF on inducing fat tissue damage during VV infection in LCMV-immune mice. We test how TNF mediates the necrosis of the fat cells, and which receptor is involved.

F. TNF and immune response

Inflammatory stimuli, such as IFN-\(\gamma\) and lipopolysaccharide (LPS), stimulate TNF production by activated macrophages/monocytes, which interacts with TNFR1 (expressed on virtually all cells in the body) and TNFR2 (expressed on immune cells, endothelia cells and adipocytes) (Aggarwal, 2003; Hotamisligil et al., 1997) and induces a variety of biological effects (Fig 1.7). TNF can stimulate macrophages and monocytes to further increase the production of TNF by these cells (Descoteaux and Matlashewski, 1990; Philip and Epstein, 1986). In addition to macrophages and monocytes, T cells, B cells and NK cells also produce TNF
Figure 1.7 Multiple actions of TNF. In response to inflammatory stimuli, activated macrophages produce TNF, which binds to its receptors and causes a variety of reactions. This figure was adapted from Bazzoni and Beutler 1996 N. Engl. J. Med. 334:1717-1725.
upon activation (Aggarwal, 2003). Since TNF receptors are present on most immune cells, TNF affects the immune system profoundly by regulating both innate and adaptive immunity.

*Innate immunity.* TNF can increase inflammation by acting on endothelial cells to induce the production of other pro-inflammatory cytokines, such as IL-1 (Beutler and Cerami, 1989), IL-6 (Jirik et al., 1989), IL-8 (Gimbrone, Jr. et al., 1989) and GM-CSF (Seelentag et al., 1987). It can also increase the migration of leukocytes into sites of inflammation by inducing changes in the shape of endothelial cells, increasing the permeability of vascular endothelium (Beutler and Cerami, 1989), and enhancing the expression of adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Doukas and Pober, 1990; Osborn, 1990). A few hours after intradermal injection of small amounts of TNF, mononuclear cells accumulate at the site (Munro et al., 1989). TNF also elevates the expression of MHC class I molecules on endothelial cells, which increases the antigen presentation to CD8 T cells to facilitate detection and elimination of target cells by antigen-specific CD8 T cells (Doukas and Pober, 1990). TNF exerts an important role in activating macrophages and monocytes. In synergy with IFN-γ, TNF increases the cytotoxicity of macrophages, which is important for protection against bacterial infection (Liew et al., 1990). Finally, NK cells are also influenced by TNF. Upon TNF stimuli, NK cells increase the expression of CD25 (high affinity IL-2 receptor)
and increase their cytolytic activity (Ostensen et al., 1987).

**Adaptive immunity.** TNF modulates antigen-specific T cells at multiple points during the response. First, TNF regulates antigen presentation by optimizing the function of dendritic cells (DC). DCs reside in many organs and tissues, and fulfill an immune surveillance function by capturing, processing and presenting antigens to lymphocytes. As shown by Yu et al., the coordinated signals from TNF and other TNF superfamily members are required for optimized maturation of dendritic cells (Yu et al., 2003). The cooperation of TNF with CD40L and RANKL, members of TNF superfamily, maximizes the ability of DC to induce virus-specific cytotoxic T lymphocytes responses.

Second, TNF also directly modulates the activation of T cells by providing co-stimulatory signals. Although resting T cells do not express TNFR, after activation by TCR engagement in the presence of secondary signals, activated T cells increase the expression of TNFR, especially TNFR2 (Zheng et al., 1995). The proliferation of activated T cells is substantially augmented by adding exogenous TNF to T cell cultures, and dramatically diminished in the presence of neutralizing antibody against TNF (Pimentel-Muinos et al., 1994). TNFR2 serves as a co-stimulator during T cell activation to increases IL-2 production by T cells, and help T cell survival by inducing the expression of anti-apoptotic molecule, Bcl-xL (Kim and Teh, 2004).

Third, TNF induces T cell death during the contraction phase of effector T cells. As shown by Singh and Suresh, the decline of CD8 T cell response against
LCMV is significantly impaired in TNF$^{-/-}$ mice long after the clearance of virus (Singh and Suresh, 2007). TNF-induced CD8 T cell death is independent of Fas, as it has different kinetics as compared to Fas-induced apoptosis, and it can not be abrogated by neutralizing antibody blockade or defective mutation (lpr) of Fas (Sarin et al., 1995; Zheng et al., 1995).

**Lymphoid tissue structure.** In addition to multiple effects on inflammation and lymphocyte activation, differentiation and death, TNF and TNF superfamily members are important for development and organization of peripheral lymphoid tissues. Mice that are lacking lymphotoxin (LT), which uses the same receptors as TNF, have no lymph nodes or gut-associated lymphoid tissues, Peyer's patches (PP). The architecture of the spleen is also severely disturbed in these mice (De et al., 1994). Compared to LT$^{-/-}$ mice, TNF$^{-/-}$ and TNFR1$^{-/-}$ mice have relatively normal development of lymphoid tissues. Although the predominant signals are transduced by LT, it was shown by Pasparakis and colleagues that TNF signaling through TNFR1 may be required for the full development of normal PP. The PP formed in TNF$^{-/-}$ and TNFR1$^{-/-}$ mice are fewer in number and smaller than the ones in wild type (WT) mice, and B cell follicles and follicular dendritic cells (DC) networks are absent in the lymph node and PP in these mice (Pasparakis et al., 1997).

Therefore, TNF modulates the immune system at a variety of levels, including inflammation, cell trafficking, activation, differentiation, proliferation, death and the development of lymphoid tissue.
**G. TNF and infection**

Considering the many effects TNF has on regulating immune responses, there is no surprise that TNF has a key role in mediating host defense against pathogens (Schluter and Deckert, 2000).

*Bacterial infection.* An essential role for TNF in immunity to bacterial infection has been demonstrated by using blocking antibodies and genetically deficient animals. As mentioned above, TNF enhances the cytotoxic pathway of macrophages, including the production of nitric oxide, which is toxic to bacteria (Vassalli, 1992). For example, in *Listerial monocytogenes* infected mice, the signaling of TNF through TNFR1 is absolutely required for controlling bacterial infection, since the absence of either TNF (Havell, 1989; Nakane et al., 1988) or TNFR1 (Pfeffer et al., 1993; Rothe et al., 1993) leads to uncontrolled bacterial growth and rapid death due to the failure of the innate immune system. TNF is also important to control infection with mycobacteria. Neutralizing TNF greatly impairs the formation of granulomas, which contain macrophages, and results in bacterial overgrowth and death of the hosts (Flynn et al., 1995; Garcia et al., 1997; Kindler et al., 1989). There is evidence in humans that anti-TNF leads to increased risk of reactivation of tuberculosis (see Discussion).

*Viral infection.* In vitro studies have suggested a direct antiviral effect of TNF,
e.g. treatment with recombinant TNF increases resistance to viral infections in some cell types, especially in the presence of IFN-γ (Mestan et al., 1986; Wong and Goeddel, 1986). Additional studies have demonstrated that TNF, mostly in synergy with IFN-γ, provides antiviral activity in vivo. For instance, during hepatitis B virus infection, TNF and IFN-γ mediate the clearance of the virus (Guidotti et al., 1996). A polymorphism of the TNF promoter, which may influence the production of TNF, has shown to be associated with defective viral clearance and establishment of chronic HBV infection (Hohler et al., 1998). TNF also interferes with the infection of human immunodeficiency virus type 1 (HIV-1), as it can block the entry of HIV-1 by directly or indirectly down-regulating the expressions of CD4 and CCR5, the receptors for HIV-1 (Di et al., 1998; Herbein et al., 1996; Karsten et al., 1996).

In this thesis, my primary focus is on the antiviral activity of TNF during VV infection. Localized expression of TNF can accelerate the clearance of VV (Lidbury et al., 1995; Ruby et al., 1997; Sambhi et al., 1991). It has also been shown that both TNFR1 and TNFR2 are required for mediating protection against VV and ectromelia virus (EV), another poxvirus. Both TNFR1−/− and TNFR2−/− mice show impaired clearance of VV and EV, and increased susceptibility to lethal EV infection (Chan et al., 2003; Ruby et al., 1997). To counteract the antiviral effect of TNF, VV encodes proteins to disrupt TNF-signaling. For example, some strains of VV encode soluble and cell-surface TNFR, which contributes to the virulence of
the virus (Reading et al., 2002). The Western Reserve (WR) strain of VV, which we are using in our studies, encodes a protein (B13R) that blocks apoptosis induced by TNF and anti-Fas antibodies (Kettle et al., 1997). TNF overcomes the blocking-effects of VV by mediating another pathway, which induces necrotic-like cell death, to eliminate the infected cells (Li and Beg, 2000). Considering its important immune regulatory and antiviral roles, we tested the effect of TNF on heterologous immunity. This thesis research directly tested the importance of TNF in protecting against VV by comparing VV loads of WT and TNF-deficient (TNF−/− or etanercept-treated) naïve mice after VV infection. We also examined the role for TNF in mediating heterologous protective immunity by using the mouse heterologous immunity model between LCMV and VV.

H. Association of TNF and TNFR with autoimmune diseases

Despite the important role TNF has in the immune system, inappropriate expression of TNF can be detrimental to the host. TNF has been implicated in many autoimmune diseases. Besides erythema nodosum, type II diabetes mellitus is thought to be associated with TNF production. As mentioned above, fat cells produce TNF, which in turn regulates the metabolism of fat cells. This cytokine has been shown to induce insulin resistance, as neutralization of TNF can increase the insulin sensitivity in rats (Hotamisligil et al., 1993), and TNF−/−
mice are protected from obesity-induced insulin resistance (Uysal et al., 1997). TNF has also been reported to be involved in the pathogenesis of RA and Crohn’s disease, a type of inflammatory bowel disease, and anti-TNF drugs have been approved for the treatment of Crohn’s disease and RA in human (Aggarwal, 2003).

Additionally, associations between a polymorphism of TNF receptors and human autoimmune diseases have been reported. A study of several families with dominantly inherited auto-inflammatory syndromes suggested an association of TNFR1 mutants with these syndromes. These TNFR1 mutants have impaired cleavage following stimulation, resulting in high levels of membrane-bound but diminished soluble form of TNFR1, which is important for down regulating TNF signal (McDermott et al., 1999). A polymorphism in TNFR2 has been shown to be associated with familial RA (Dieude et al., 2002) and systemic lupus erythematosus (Komata et al., 1999; Morita et al., 2001). This polymorphism (TNFR2 196R), with a methionine replaced by an arginine, is a more effective transducer for the signal of TNF (Morita et al., 2001).

As TNF has been implicated in the pathogenesis of EN, a form of panniculitis in humans, we tested here whether TNF was responsible for the generation of panniculitis in VV-infected LCMV-immune mice. We also explored the pathway that TNF used to induce this pathology.
I. Thesis objectives

During VV infection, LCMV-immune mice show rapid viral clearance, but develop panniculitis on the visceral fat pads. According to previous studies, TNF may play a role in host defense against VV, and it has been suggested to be involved in the pathogenesis of EN, the most common form of panniculitis in humans. Thus, our hypothesis is that TNF, like IFN-γ, is required for mediating both heterologous protection and panniculitis in LCMV-immune mice infected with VV. My thesis is presented in three parts:

Chapter III: Heterologous immunity can abrogate the requirement for TNF in protection against VV.

This chapter examines the role of TNF in mediating protection against VV in both naïve and LCMV-immune mice by comparing VV titers between VV-infected WT and TNF-deficient (TNF⁻/⁻ or etanercept-treated) mice.

Chapter IV: Individual variations in the pathogenesis of viral infection are determined by private specificity.

By transferring the same population of splenocytes from one LCMV-immune donor into two naïve recipients, this chapter demonstrates the major determinant of individual variations in the pathogenesis of panniculitis in LCMV-immune mice during VV infection. It also provides an approach to minimize the influence of
individual variation on the prediction of the results from immunopathology studies in the next chapter.

**Chapter V :** TNF/LT and FasL mediate the pathogenesis of panniculitis in LCMV-immune mice during VV infection.

This chapter illustrates whether TNF and LT induce the necrosis of fat tissues in LCMV-immune mice during VV challenge, by comparing the levels of panniculitis between TNF-deficient mice and WT controls.
CHAPTER II: MATERIALS AND METHODS

A. Mice

Male C57BL/6 (B6, H2b) mice, TNFR1/- mice (B6.129-Tnfrsf1a<sup>tm1Mak</sup>/J), TNFR2/- mice (B6.129S2-Tnfrsf1b<sup>tm1Mwm</sup>/J), IFN-γR/- mice (B6.129S7-Ifngr1<sup>tm1Agt</sup>/J) and FasL-deficient (gld) mice (B6Smn.C3-Fasl<sup>gld</sup>/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Ly5.1 mice (B6.SJL-Ptprc<sup>a</sup>) were purchased from Taconic Farms. TNF/- mice (B6; 129S6-TNF<sup>tm1gkl</sup>) originally from Jackson Laboratory were backcrossed onto B6 mice using MAX-BAX speed congenics until the mice were 99% B6 background (Charles River Laboratories, Inc., Wilmington, MA). All mice were maintained under specific pathogen-free conditions within our animal colony.

B. Viruses

LCMV is a RNA virus in the Old World arenavirus family. The Armstrong strain of LCMV was propagated in BHK21 baby hamster kidney cells (Selin et al., 1994). Vaccinia virus (VV) is a DNA virus in the poxvirus family. The WR strain of VV was propagated in either NCTC-929 cells or Hela cells (Selin et al., 1994).
C. Infection protocol and blocking treatments for TNF and TRAIL

For LCMV-immunization, mice were inoculated IP with $5 \times 10^4$ PFU of LCMV Armstrong. In most experiments, immune mice were challenged with $1 \times 10^6$ PFU of VV IP six weeks after immunization, when the immune system had returned to homeostasis. For experiments using gld mice, all the mice were immunized with LCMV at 4 weeks of age and challenged with VV four weeks later. For experiments using TNF$^{-/-}$ mice, both KO mice and B6 controls were challenged with $7.5 \times 10^5$ PFU VV IP. For the mortality test in the IP model, various VV doses were used for different groups, as indicated in the context. For the mortality test in the IN model, metofane-anesthetized mice were immunized with $5 \times 10^5$ PFU LCMV Armstrong IN, and 6 weeks later both immune and non-immune mice were challenged $4 \times 10^4$ PFU VV IN.

For experiments blocking TNF and LT in vivo, 2 days before infection, mice were treated IP with either 100$\mu$g of TNFR2:IgG fusion protein (etanercept, Immunes Corporation, Thousand Oaks, CA, a gift from Dr. Dale Greiner) or 100$\mu$g of goat anti hIgG-Fc (Fitzgerald, Concord, MA), as a control. For TRAIL blockade, mice were given 250$\mu$g of anti-mouse TRAIL antibody (109308, Biolegend) IP at day -1 and day 3 of VV infection.
D. Synthetic peptides

LCMV-specific peptides: NP396-404 (FQPQNGQFI; D<sup>b</sup> binding), GP33-41 (KAVYNFATC; D<sup>b</sup> binding), GP276-286 (SGVENPGGYCL; D<sup>b</sup> binding), NP205-212 (YTVKYPNL; K<sup>b</sup> binding), GP118-125 (ISHNFCNL; K<sup>b</sup> binding); VV-specific peptides: B8R20-27 (TSYKFESV; K<sup>b</sup> binding), E7R130-137 (STLNFLNL; K<sup>b</sup> binding) and A11R198-205 (AVNYANL; K<sup>b</sup> binding) were purchased from BioSource International, and were purified with reverse phase-HPLC to 90% purity.

E. Plaque assay

VV loads in fat pads and testes were titrated on ATCC vero cell monolayers using a 3-day plaque assay. Tissues, after extraction from VV-infected mice, were put in 1mL complete RPMI 1640 medium (GIBCO, Gaithersburg, MD). Homogenized tissues were centrifuged at 2,000rpm for 20 minutes at 4°C, and supernatant stored at -80°C. Frozen aliquots were thawed on the day of plaque assay (day 0) and immediately applied on vero cells grown in 6-well plates (Costar, Cambridge, MA). Ten-fold serial dilutions of tissue homogenate (100 μL) were added onto the cells in 1 mL complete MEM medium (GIBCO, Gaithersburg, MD) containing 10% heat-inactivated FBS, 50units/mL penicillin and 50μg/mL streptomycin. Cells were incubated at 37°C for 90 min. Then 4 mL overlay mixture
(1:1 mixture of 1% SeaKem® ME agarose (FMC, Rockland, ME) and EMEM (Lonza, Walkersville, MD) containing 10% FBS) was overlaid onto the cells. Plates were kept in a 5% CO₂-humidified atmosphere at 37°C for two days. At day 2 of assay, the plates were stained with 2 mL of the overlay mixture containing 0.1% neutral red (Sigma). Plaques were counted on day 3. Virus titers were shown as log₁₀ of PFU per whole organ. Results were expressed as the geometric mean titers, i.e., the arithmetic averages of the logs for four or five separate animals titrated for virus individually ± SEM.

**F. Preparation of leukocytes**

Spleens were harvested and ground between the frosted ends of two microscope slides. The cell suspension was then passed through a fine nylon mesh to obtain single cell suspension. Cells from the peritoneal cavity were collected by lavaging with 10 mL cold RPMI 1640 medium. Infiltrating leukocytes were isolated from fat pads by mincing and digesting with 0.5 mg/ml type II collagenase (Sigma-Aldrich, St. Louis, MO) and 100 U/ml type I DNase (Sigma-Aldrich) in HBSS buffer (GIBCO, Gaithersburg, MD) plus 10% heat inactivated FBS for 45 minutes at 37°C. The digest mixture was then centrifuged at 2,000 rpm for 10 minutes. Cell pellets were suspended in RPMI and passed through mesh to obtain single cell suspensions, which then were layered over
lympholyte-M (Cedarlane Labs, Hornby, Canada) and centrifuged at 2,000 rpm for 20 minutes at room temperature. The mononuclear band was collected and washed with RPMI 1640 medium to remove lympholyte-M. Contaminating erythrocytes were removed from the leukocyte preparations by treatment with 0.84% NH₄Cl.

G. Flow cytometry and intracellular IFN-γ staining

Isolated cells (2X10⁶) were incubated in 96-well plates (5h; 37°C) with 5 μM synthetic peptide, 10 U/mL human rIL-2 (BD Bioscience), and 0.2 μL of GolgiPlug (BD Bioscience). Cells were then washed in FACS buffer (PBS, 2% FCS, and 0.1% NaN₃), blocked with anti-Fc antibody (2.4G2, BD Biosciences) for 15 minutes at 4°C. After two washes with FACS buffer, cells were incubated (30 min; 4°C) with a combination of fluorescently labeled mAbs specific for CD44 (IM7, FITC, BD Bioscience), CD8α (53-6.7, PerCP-Cy5.5, BD Bioscience) and CD45.1 (A20, PE-Cy7, eBioscience). Cells were then fixed and permeabilized with BD Cytofix/Cytoperm™ solution (51-2090KZ, BD Bioscience) for 20 minutes at 4°C, and washed with 1X perm/wash™ solution (51-2091KZ, BD Bioscience). To detect cytokine production, cells were stained with mAb specific for IFN-γ (XMG1.2, APC, BD Bioscience) and TNF (MP6-XT22, PE, BD Bioscience). The samples were analyzed using a BD Biosciences LSR II and FlowJo software. The total number of
epitope-specific CD8 T cells was calculated by multiplying the percentage with the total cell yield from individual tissue. For most experiments, the lymphocytes isolated from peritoneal cavity and fat pads were pooled for each group, due to the low cell yield from these organs.

**H. Adoptive transfer of LCMV-immune splenocytes**

Ly5.1 mice were inoculated IP with 5X10⁴ PFU of LCMV Armstrong and were considered immune more than 6 weeks after infection. For paired adoptive transfer experiments, whole splenocytes from one Ly5.1 LCMV-immune mouse were equally split and adoptively transferred intravenously (IV) into two hosts (two Ly5.2 C57BL/6J mice or one Ly5.2 C57BL/6J mouse and one Ly5.2 TNFR⁻⁻ mouse) in a 200-μL volume of HBSS. For pooled adoptive transfer experiments, pooled splenocytes from five or more Ly5.1 LCMV-immune mice were equally transferred IV into four or five Ly5.2 C57BL/6J mice in a 200-μL volume of HBSS. One day post transfer, all recipients were challenged IP with 1 X 10⁶ PFU WR strain of VV.

**I. CFSE labeling**

Splenocytes from immune donor mice were harvested and isolated as described above. In some experiments, the donor immune cells were labeled with
CFSE by suspending at $2.0 \times 10^7$ cells/ml in HBSS containing 2 µM CFSE (Molecular Probes) and incubated for 12 min at 37°C. After incubation, donor cells were washed twice with HBSS.

**J. Scoring the levels of panniculitis**

The level of panniculitis was score visually based on the severity of disease from level 0 to 8. Levels 1 and 2 represent very mild to mild disease with a few white necrotic spots on one or both lower abdominal fat pads; levels 3 and 4 represent mildly moderate and moderate with larger patches of necrosis of the lower abdominal fat pads and extension into the upper left quadrant fat pad around the spleen; levels 5 and 6 represent moderately severe to severe with very extensive large patches of necrosis throughout visceral fat pads as well as the splenic fat pad; level 7 represents very severe disease with such severe fatty necrosis that the organs are adherent to each other; and level 8 represents moribund with the fatty necrosis so severe that the animal can not survive.

**K. 3T3-L1 cell line and TNF stimulation**

3T3-L1 fibroblasts were maintained in 150-mm tissue culture dishes by seeding at a density of 750,000-1,000,000 cells per dish. Cells were grown to
confluence in complete media (high glucose DMEM (Gibco) containing 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin) and kept in a 10% CO₂-humidified atmosphere at 37°C. Medium was changed every 2-3 days. Two days after the fibroblasts achieved confluence, differentiation to adipocytes was induced by incubating the cells for 3 days in 25mL complete media containing 4 μg/mL insulin (Sigma), 0.25 μM dexamethasone (Sigma) and 0.5 mM 3-isobutyl 1-methylxanthine (Sigma). Then media was replaced with regular complete media. Four days after differentiation, cells were harvested and resuspended in 50mL complete media. 1mL of resuspended cells were distributed per well of a 12-well plate. After 48 hours, the adipocytes were pretreated for one hour with 1 μg/mL hamster IgG (20003, Alpha Diagnostics international Inc, San Antonio, TX), TNFR1 antagonistic antibody (MAB430, R&D system) or TNFR2 antagonistic antibody (113301, Biolegend), followed with 24-h stimulation with 5ng/mL TNF (654245, Calbiochem).

L. Isolation of RNA and quantitative real-time PCR

RNA isolation was performed according to the RNeasy mini kit protocol (Qiagen). The concentration and the purity of the RNA were determined by measuring the absorbance at 260/280 nm. cDNA was synthesized using 1 μg of RNA and the iScript cDNA synthesis kit (Bio-rad) for a 20-μL reaction volume. For
real-time PCR, 1 μL of synthesized cDNA was put into one well of a 96-well plate for detections of Fas mRNA (forward primer 5’-TAT CAA GGA GGC CCA TTT TGC-3’ and reverse primer 5’-TGT TTC CAC TTC TAA ACC ATG CT-3’); and DR5 mRNA (forward primer 5’-TCT GCC AGT CAT GCT CTA ACT G-3’ and reverse primer 5’-CTG AGT CTT GCC AGG TT C CGT GT-3’). The expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) did not change over a 24-hour period TNF stimulation, so it was used as an internal loading control (forward primer 5’-TCA GTC AAC G GG GGA CAT AAA-3’ and reverse primer 5’-GGG GCT GTA CTG CTT AAC CAG-3’). 10 pmol of forward and reverse primer was added to each well along with DNase/RNase free water and 25 μL of iQ SYBR Green Supermix (Bio-rad) for a 50-μL final volume. Samples were run on the MyIQ Real time PCR System (Bio-rad). Relative gene expression was determined by using the 2^-ΔΔCT method (Livak and Schmittgen, 2001). The real-time PCR program was: 95°C for 3 minutes, followed by 95°C for 15 seconds, 60°C 30 seconds and 72°C 30 seconds for a total of 40 cycles, and a 5-minute incubation at 72°C.

M. Histology analysis

Histology was done on fat pads isolated at 6 days after VV challenge. Samples were fixed in 10% neutral buffered formaldehyde and then
paraffin-embedded. Tissue sections (5 μm) were stained with hematoxylin and eosin by Diabetes and Endocrinology Research Center Morphology Core Laboratory (DERC lab).

N. Statistical analysis

Results were expressed as the mean ± SEM. The Wilcoxon signed rank test and Mann-whitney test were used for analysis of T cell recruitment data and relative weight lost. The Log rank test was used for analyzing survival curves. Intra-class correlation was used to analyze panniculitis data from paired transfer experiments. Student's t test was used for analysis as indicated in the context. P values (2-tailed) less than 0.05 were considered statistically significant.
CHAPTER III:
HETEROLOGOUS IMMUNITY CAN ABROGATE THE
ROLE OF TNF IN PROTECTION AGAINST VACCINIA VIRUS

TNF is a pivotal cytokine in host defenses and exerts a broad range of effects on the innate and adaptive immune systems. It is not surprising that disruption of TNF signaling pathways in animal models leads to increased susceptibility to a variety of pathogens, such as viruses, bacteria and fungi (Herbein and O’Brien, 2000; Schluter and Deckert, 2000). Interestingly, clinical trials involving TNF-blocking reagents used in a number of autoimmune diseases have generally not demonstrated increased susceptibilities to infections which may have been predicted. Post-marketing analysis has demonstrated some limited increased susceptibility, with sporadically reported cases of infections, particularly by Mycobacterium tuberculosis, occurring in patients treated with TNF-blocking reagents (Calabrese et al., 2004; Desai and Furst, 2006).

In mouse studies, experimental viral immunologists go to great lengths to assure that their animal colonies are free of endogenous pathogens in order to design reproducible experiments. These experimental results are then thought to provide the basis for our understanding of the human immune responses to
viruses. Although these findings can be enlightening, humans are not immunologically naïve, and they often have memory T cell populations which can cross-react with and respond to a new related or unrelated infectious agent and cause a dramatic difference in disease course, a phenomenon known as heterologous immunity. Heterologous immunity can influence protective immunity, immunopathology and/or immune deviation of cytokine-producing T cell subsets (Welsh and Selin, 2002) and has been well documented in mouse models, as well as during human infections (Clute et al., 2005; Urbani et al., 2005; Wedemeyer et al., 2001; Welsh et al., 2004). For instance, LCMV-immune mice are partially protected against infection with VV, as compared to infected naïve mice (Chen et al., 2001; Selin et al., 1998). Cross-reactive LCMV-specific memory T cells are activated by VV infection and participate in mediating this protective effect (Cornberg et al., 2007; Kim et al., 2005; Cornberg et al., manuscript submitted).

TNF, a cytokine which is involved in the recruitment and activation of immune cells at sites of infection, can act synergistically with IFN-γ to interfere with replication of viruses such as VV (Herbein and O'Brien, 2000; Wong et al., 1988). Studies using TNFR−/− mice or recombinant VV expressing the TNF gene have demonstrated an important role for TNF in controlling the replication of VV in a naïve host (Chan et al., 2003; Lidbury et al., 1995; Ruby et al., 1997; Sambhi et al., 1991).

By comparing VV loads between TNF-deficient (either neutralizing antibody
treatment or genetic KO) and WT VV-infected mice, we directly demonstrated the role of TNF in mediating protection against VV in naïve mice. We also attempted to understand the requirement for TNF in mediating heterologous immunity in LCMV-immune mice, and explain the difference in mouse versus human studies concerning the importance of TNF in mediating protection against intracellular pathogens.

A. **TNF is not required for expansion, recruitment and skewing of LCMV-specific CD8 memory T cells at the site of VV infection.**

It has already been demonstrated that LCMV-immune mice have enhanced recruitment and activation of cross-reactive LCMV-specific memory CD8 T cells into infected peripheral organs, such as the lung after IN inoculation or the peritoneal cavity, after IP inoculation of VV (Chen et al., 2001; Kim et al., 2002; Selin et al., 1998). We questioned whether the presence of TNF was required for this recruitment and activation of cross-reactive LCMV-specific memory T cells. We began these studies by examining the accumulation of LCMV-specific CD8 T cells at the sites of infection, including the peritoneal cavity and abdominal fat pads, after IP infection with VV in LCMV-immune TNF−/− mice. It should be noted that the TNF−/− mice generated normal LCMV-specific T cell responses and were able to clear LCMV, as shown in previous reports (Klavinskis et al., 1989; Leist and
Zinkernagel, 1990). The lymphocytes from the peritoneal cavity and fat pads were harvested from both infected and uninfected animals at day 6 of VV infection, which is the peak of the VV-specific T cell response and a time point when we would expect antigen-specific cells, not bystander memory cells, to accumulate in infected peripheral sites (Ely et al., 2003). Due to low cell yields from the peritoneal cavity and fat pads, lymphocytes isolated from the same group of five mice were pooled. Cells were stimulated with LCMV-specific peptides and examined for IFN-γ production by intracellular cytokine staining (ICCS). We calculated the total number of LCMV-specific CD8 T cells by combining the total response to five well-defined LCMV class-I epitopes (NP396-404, GP33-41, GP276-286, NP205-212, GP118-125). In Figure 3.1A, a representative experiment demonstrated that the total number of LCMV-specific CD8 T cells increased 9- and 11-fold in the peritoneal cavity and 117- and 243-fold in the fat pads of the WT and TNF−/− LCMV-immune mice, respectively, upon VV challenge. Thus, TNF alone does not play a role in mediating expansion and recruitment of cross-reactive LCMV-specific CD8 T cells into infected peripheral organs.

In order to rule out that lymphotoxin (LT), which has redundant functions with TNF and uses the same receptors (Aggarwal et al., 1985), could have an effect on the recruitment of LCMV-specific CD8 T cells, we utilized the human TNFR II:IgG fusion protein, etanercept. Etanercept blocks the activity of both TNF and LT, and it is used clinically for the treatment of patients with RA and Crohn’s disease.
Figure 3.1 TNF and LT were not required for the expansion and recruitment of LCMV-specific CD8 T cells. WT, TNF-deficient (TNF−/−) or etanercept-treated) and IFNγR−/− LCMV-immune mice were challenged with VV IP or uninfected. PEC and fat pad lymphocytes were isolated 6 days after VV infection, and examined by ICCS. Lymphocytes from WT and TNF-deficient mice were pooled. A shows a representative experiment using TNF−/− mice. B summarizes the results from all the experiments using either TNF−/− mice (solid) or etanercept-treatment (open). TNF+: WT mice; TNF−: TNF-deficient; LCMV-imm: LCMV-immune mice; LCMV-imm+VV: LCMV-immune mice infected with VV. The same symbols represent results from the same experiment. IFNγR−/− experiments were done separately. Bars represent the mean of each group. n=5 per group, the Wilcoxon signed rank test was used for statistical analysis.
(Aggarwal, 2003; Feldmann and Maini, 2001). Two days prior to VV infection, LCMV-immune WT mice were treated with either 100 μg etanercept or 100 μg IgG Fc, as a control. Figure 3.1B, which summarized the results from experiments using either etanercept treatment or TNF−/− LCMV-immune mice, demonstrated a significant 14-fold (range: 8- to 29-fold) and 9.5-fold (range: 6- to 11-fold) increase in LCMV-specific CD8 T cell numbers in the peritoneal cavity of WT and TNF-deficient mice respectively, upon VV infection. Although there were more variations between experiments, the numbers of LCMV-specific CD8 T cell also increased from 0.8- to 163-fold and from 3- to 534-fold in the fat pads of the wild type and TNF-deficient LCMV-immune mice respectively, upon VV infection.

As shown previously by our group, the protective immunity against VV provided by LCMV immunity is abrogated in IFN-γR−/− mice. It was tested whether this loss of protection was due to an inability of recruiting LCMV memory cells into peripheral organs in the absence of IFN-γ. Like TNF-deficient mice, the accumulation of LCMV-specific CD8 T cells was normal in IFNγR−/− mice. LCMV-immune IFNγR−/− mice had equivalent numbers of LCMV-specific memory CD8 T cells in the peritoneal cavity and fat pads at day 6 after infection with 1X10^6 PFU VV IP as control WT mice (Fig 3.1B).

We have previously reported that each individual LCMV-immune mouse upon VV infection selectively expands a different cross-reactive LCMV epitope-specific memory CD8 T cell population, leading to different patterns of skewed
LCMV-specific responses. This variation in skewing is dependent upon the private specificity of the T cell repertoire of each immune mouse (Kim et al., 2005). We questioned whether TNF could impact the activation of these cross-reactive memory T cell responses by assessing selective expansion of LCMV epitope-specific memory T cells. Compared with control mice, the absence of TNF did not impact the skewing of LCMV-specific CD8 memory responses during VV infection (Fig 3.2 and 3.3). After LCMV immunization, TNF-deficient mice developed similar LCMV-specific response as WT mice (Fig 3.2A and 3.3A). The two representative WT LCMV-immune control mice demonstrated a preferential expansion of LCMV-NP205- or GP33/34-specific T cells (Fig 3.2B). The LCMV-immune TNF−/− mice demonstrated similar patterns of skewing, with one mouse preferentially expanding both GP33/34- and NP205- specific T cells while the other two mice expanded only one of these populations (Fig 3.3B). Different patterns of skewing in LCMV-specific response were also evidenced in IFN-γR−/− mice. As shown in figure 3.4A, four LCMV-immune mice showed very similar hierarchies of LCMV-specific responses. However, after VV infection, the hierarchies of LCMV-specific responses were variable between individuals (Fig 3.4B). Thus, neither TNF/LT nor IFN-γ affects the activation and selective expansion of cross-reactive CD8 T cells.

As shown previously by our group, protection against VV in LCMV-immune
Figure 3.2 During VV infection, selective activation and expansion of cross-reactive LCMV-specific memory CD8 T cells resulted in skewed LCMV response. LCMV-immune C57BL/6J mice were tail bled and analyzed for LCMV-specific CD8 T cell responses. Six days post VV challenge, splenocytes were isolated and evaluated for the evidence of selective expansions of LCMV epitope-specific response during VV infection consistent with cross-reactivity. Normal hierarchy of LCMV CD8 T cell memory response is shown in A, and VV-induced skewed LCMV-specific responses are shown in B. Two representatives of a total of twenty VV-infected LCMV-immune mice are shown in B.
Figure 3.3 TNF was not necessary for the selective activation and expansion of cross-reactive LCMV-specific memory CD8 T cells. LCMV-immune TNF−/− mice were tail bled and analyzed for LCMV-specific memory CD8 T cell response. Six days post VV challenge, lymphocytes were isolated from spleens and evaluated for the evidence of selective expansions of LCMV epitope-specific CD8 T cell response during VV infection consistent with cross-reactivity. Normal hierarchy of LCMV CD8 T cell memory response in TNF−/− mice is shown in A, and VV-induced skewed LCMV-specific responses are shown in B. Three representatives of twenty VV-infected LCMV-immune TNF−/− mice are shown in B.
Figure 3.4 Hierarchies of LCMV-specific CD8 T cell responses in IFN-γR−/− mice with or without VV challenge. Figure shows the hierarchies of LCMV-specific CD8 T cell responses with or without VV challenge in LCMV-immune IFN-γR−/− mice. PEC were harvested from uninfected controls (A) and VV day-6 infected mice (B), and then examined by ICCS. The responses against these five LCMV epitopes were normalized as 100%. The responses from the same mouse were linked by lines.
mice is mediated by both CD4 and CD8 LCMV-specific memory T cells (Selin et al., 1998). After TCR-antigen engagement early after VV infection, cross-reactive T cells are activated and produce IFN-\(\gamma\) (Chen et al., 2001), a cytokine to which VV is very sensitive. Then, activated cross-reactive LCMV-specific T cells migrate to the site of infection and mediate more rapid clearance of VV. As shown above, neither TNF/LT nor IFN-\(\gamma\) played a role in the activation and accumulation of cross-reactive LCMV-specific CD8 T cells into sites of VV replication. However, heterologous protective immunity against VV is not evident in either IFN-\(\gamma\)R\(^{-/-}\) mice or in mice treated with IFN-\(\gamma\) blocking antibody (Selin et al., 1998), indicating an indispensable role for IFN-\(\gamma\) in protection against VV in LCMV-immune mice. Additionally, there is some evidence suggesting TNF is also required for efficient clearance of VV in naïve mice (Lidbury et al., 1995; Ruby et al., 1997; Sambhi et al., 1991). We next questioned whether TNF had effects on mediating heterologous protective immunity against VV in LCMV-immune mice.

B. In the absence of TNF, prior immunity to LCMV provides efficient protection and abrogates the role of TNF during VV infection.

To understand the role TNF plays in controlling VV infection, we first tested whether TNF-deficient mice could generate normal VV-specific responses. Figure
3.5 shows the experimental protocol, where naïve and LCMV-immune WT and TNF-deficient mice were challenged with VV. At day 6 of VV infection, the VV-specific response was examined on splenocytes by ICCS. Naïve and LCMV-immune etanercept-treated mice generated similar levels of response against VV as their IgG-treated controls (Fig 3.6). Similar results were also seen in TNF−/− mice after VV challenge (data not shown).

We next examined whether recruited LCMV-specific memory CD8 T cells could mediate protective immunity against VV in TNF-deficient mice. The body weight of each individual mouse was recorded daily, after VV infection. As shown in Figure 3.7A, non-immune TNF−/− mice lost significantly more weight than WT non-immune mice after VV infection, consistent with previous reports (Lidbury et al., 1995; Ruby et al., 1997; Sambhi et al., 1991), suggesting that TNF was important for controlling VV in naïve mice. Both LCMV-immune WT and TNF−/− mice had only minimal weight loss, in contrast to their non-immune controls (Fig 3.7A), suggesting TNF−/− LCMV-immune mice also had protective immunity against VV. To more directly demonstrate whether TNF was required for mediating the heterologous protective immunity against VV, the VV titers of the fat pads and testes at day 6 post VV infection were determined by plaque assay. Viral loads were significantly decreased in TNF−/− LCMV-immune mice compared to naïve TNF−/− controls (Fig. 3.8A; Table 3.1), indicating that the heterologous protective immunity against VV provided by LCMV immunity was not dependent on TNF. Our data directly showed that there was a significant 11-fold increase in VV titers in
A. Experiment with TNF^{-/-} mice

<table>
<thead>
<tr>
<th>Naïve</th>
<th>LCMV-immune</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>TNF^{-/-}</td>
</tr>
</tbody>
</table>

7.5 \times 10^5 PFU

B. Experiment with TNF blockade

<table>
<thead>
<tr>
<th>Naïve</th>
<th>LCMV-immune</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>C57BL/6J</td>
</tr>
</tbody>
</table>

IgG Fc | Etanercept | IgG Fc | Etanercept

Wild type | TNF-deficient | Wild type | TNF-deficient

Day -2

1.0 \times 10^6 PFU

Figure 3.5 Experimental design to test the role of TNF and LT in mediating heterologous protective immunity against VV.
Naïve mice infected with VV (Day 6 spleen)

<table>
<thead>
<tr>
<th>IgG-Fc</th>
<th>Etanercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>8.6</td>
</tr>
</tbody>
</table>

LCMV-imm mice infected with VV (Day 6 spleen)

<table>
<thead>
<tr>
<th>IgG-Fc</th>
<th>Etanercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Figure 3.6 TNF-deficient mice had comparable levels of VV-specific response as WT controls. Naïve and LCMV-immune IgG or Etanercept treated mice were infected with VV. At day 6 of infection, splenocytes were harvested and stimulated with VV-infected DC2.4 for 5 hours, and then stained for CD8, CD44 and intracellular IFN-γ production. Cells were gated on CD8⁺ lymphocytes. One representative mouse from each group is shown.
Figure 3.7 Immunity to LCMV protected TNF-deficient mice from losing weight during VV infection. WT or TNF-/- LCMV-immune mice and age matched naïve controls (A); IgG- or etanercept-treated LCMV-immune mice and age matched naïve controls (B) were challenged with VV. Relative weights are shown. n=4 or 5 per group; A Mann-whitney test was used for statistical analysis, **, p < 0.01. Graphs show the averages of each group ±SEM. A shows a representative of four experiments with similar results; B shows a representative of three experiments with similar results.
Figure 3.8 Previous immunity to LCMV compensated for the absence of TNF and LT in protection against VV infection. WT or TNF−/− LCMV-immune mice and age matched naïve controls (A), IgG or etanercept-treated LCMV-immune mice and age matched naïve controls (B) were challenged with VV. Viral titers in fat pads and testes were determined 6 days post VV infection by plaque assay. n=4 or 5 per group; student’s t test was used for statistical analysis, P value is indicated, if p>0.05; *, p ≤ 0.05; **, p < 0.01, ***, p < 0.001. Graphs show the averages of each group and the error bars represent ±SEM. A shows a representative of four experiments with similar results; B shows a representative of three experiments with similar results.
### Table 3.1 Immunity to LCMV decreased VV titers in the absence of TNF and LT

**Mean (±SEM) Log10 of VV titer in the Fat Pad**

<table>
<thead>
<tr>
<th>Exp</th>
<th>TNF+ Naïve +VV</th>
<th>TNF+ LCMV-imm+VV</th>
<th>% of inhibition</th>
<th>TNF- Naïve +VV†</th>
<th>TNF- LCMV-imm+VV‡</th>
<th>% of inhibition</th>
</tr>
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<td>97.5</td>
<td>4.9±0.1 (+1.1)</td>
<td>1.6±0.6 (-0.6)</td>
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<td>4.1±0.3# (+0.7)</td>
<td>2.3±0.3ıt (±0.2)</td>
<td>92.9±4.1</td>
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**Mean (±SEM) Log10 of VV titer in the Testis**

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<tr>
<th>Exp</th>
<th>TNF+ Naïve +VV</th>
<th>TNF+ LCMV-imm+VV</th>
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<th>TNF- LCMV-imm+VV‡</th>
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<td>1.9±0.3ıt (±0.3)</td>
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</tbody>
</table>

Fat pads and testes were titrated at day 6 of VV infection. VV titers differed between experiments due to the use of different viral stocks (n=4 or 5 mice/group). Numbers in parentheses represent the log change compared to †TNF+ naïve+VV or ‡TNF+ LCMV-imm+VV mice. Statistically significantly different from #TNF+ naïve+VV mice (Student’s paired t test, p<0.05). or §TNF- naïve+VV mice (p<0.01). † No significant difference between TNF+ and TNF- LCMV+VV mice (Student paired t test, p=0.5 for FP and p=0.4 for testes). UND, undetectable.
TNF−/− non-immune mice in comparison to non-immune WT mice (Fig. 3.8A; Table 3.1). In contrast, the TNF−/− LCMV-immune mice controlled VV replication as efficiently as WT LCMV-immune mice, suggesting that the prior immunity to LCMV made TNF less important in protection against VV infection (Fig. 3.8A; Table 3.1).

To rule out the possibility of LT compensating for the lack of TNF, we used etanercept treatment to block both TNF and LT function. These studies recapitulated the findings in the TNF−/− mice. Etanercept-treated LCMV-immune mice were comparable to WT LCMV-immune mice showing significantly less weight loss, and more rapid virus clearance upon VV infection (Fig. 3.7B; 3.8B and Table 3.1) than IgG-treated or etanercept-treated naïve controls.

The efficient clearance of VV in LCMV-immune mice was observed in all the experiments using TNF−/− mice or etanercept treatment, with WT and TNF-deficient (TNF−/− and etanercept-treated) LCMV-immune mice showing an average of 85% and 91-92% lower VV titers in fat pads and testes than their naïve controls, respectively (table 3.1). While, the VV loads in TNF-deficient naïve mice were almost a log higher than those in WT naïve mice, after VV infection. These data indicate that TNF played a role in protection against VV in naïve mice, but neither TNF nor LT was needed for mediating heterologous protective immunity against VV in LCMV-immune mice. In fact, the prior immunity to LCMV can abrogate the role TNF plays in defending against VV in naïve mice.
C. Prior immunity to LCMV protects TNF-deficient mice from lethal VV infection.

According to the data shown above, it seemed that TNF/LT was not important for mediating heterologous protective immunity against VV. We then tested whether LCMV-immunity itself would be enough to protect against lethal VV infection in the absence of TNF signaling. Previous studies have shown that the protective immunity mediated by LCMV memory T cells protect mice from a lethal VV challenge in the respiratory infection model (Chen et al., 2001). Here we demonstrated for the first time that LCMV-immune mice were also protected against a lethal IP VV challenge (Fig 3.9A). After a lethal dose VV infection, the LCMV immunity significantly (p=0.03) increased the time of median survival from 5 days in non-immune mice to 9.5 days in immune mice. To test whether immunity to LCMV could protect the host from a lethal VV infection in the absence of TNF, LCMV-immune TNF−/− or etanercept-treated WT mice were infected with lethal doses of VV IP. Dramatic protection was found in etanercept-treated mice. After infection with a lethal dose (4X10^6 PFU) of VV IP, all the etanercept-treated naïve mice were dead by day 7, while 50% of the etanercept-treated LCMV-immune mice survived the infection (Fig 3.9B). Figure 3.9C shows the survival of naïve and LCMV-immune TNF−/− mice after infection with 1.6X10^6 PFU VV IP. The mortality was decreased significantly from 80% in the naïve TNF−/− mice, to 45% in the immune TNF−/− mice. We were also able to demonstrate similar results in the IN
Figure 3.9 LCMV-immune mice were protected against lethal VV IP infections in the absence of TNF and LT. LCMV-immune mice (open circle) and naïve controls (solid circle) were infected with lethal dose of VV IP. The percentage of survival was recorded daily till the end of experiment. A, naïve (n=9) and LCMV-immune (n=10) WT mice were infected with 5.0 X 10^6 PFU of VV. B, naïve (n=10) and LCMV-immune (n=10) etanercept-treated WT mice were infected with 4.0X10^6 PFU of VV. C, naïve (n=10) and LCMV-immune (n=11) TNF^−/− mice were infected with 1.6X10^6 PFU VV. The Log rank test was used for statistical analysis; *, p < 0.05; ***, p < 0.001.
model of infection with VV in LCMV-immune mice. There was significantly less
mortality upon lethal VV infection in both IgG- or etanercept-treated
LCMV-immune mice (20% and 10% respectively) than the control IgG- or
etanercept-treated non-immune mice (70% and 50% respectively) (Fig 3.10). Thus,
prior immunity to LCMV could protect a host from a lethal infection with VV even in
the absence of TNF, a cytokine important in mediating protection against VV in
naïve mice.

D. Chapter summary

By using TNF−/− mice, we directly confirm the importance of TNF in defending
against VV in naïve mice; however, prior immunity to a virus such as LCMV
completely alters the picture. A LCMV-immune TNF-deficient host has a
comparable level of protection to VV as a WT LCMV-immune host, suggesting that
TNF and LT do not mediate heterologous protective immunity against VV in
LCMV-immune mice. The heterologous immunity found in these mice, most likely
through IFN-γ production by cross-reactive memory CD8 T cells, can completely
substitute for the antiviral effect of TNF and LT in protection against VV in naïve
mice. These data imply that the anti-TNF therapies to treat various painful
diseases in humans may be relatively safe perhaps in large part due to
heterologous immunity. Since humans have been exposed to many pathogens
throughout their lives, they have a large complex pool of memory T cells that have
Figure 3.10 Immunity to LCMV significantly reduced the mortality of hosts challenged with lethal dose VV IN. IgG- (A) or etanercept-treated (B) LCMV-immune mice (open circle) and naïve controls (solid circle) were infected with 4.0 X 10^4 PFU of VV IN. The percentage of survival was recorded daily till the end of experiment. The Log rank test was used for statistical analysis; n=10 per group; *, p < 0.05.
the potential to cross-react with any new pathogen and mediate heterologous protective immunity.
CHAPTER IV:

PATHOLOGICAL FEATURES OF HETEROLOGOUS IMMUNITY ARE REGULATED BY THE PRIVATE SPECIFICITIES OF THE IMMUNE REPERTOIRE

A number of studies have shown that the T cell response to viral infections may be influenced by memory T cells generated in response to unrelated pathogens (Brehm et al., 2002; Chen et al., 2001; Clute et al., 2005; Selin et al., 1998; Urbani et al., 2005). This alteration in responses is displayed by changes in T cell immunodominance hierarchies (Brehm et al., 2002; Kim et al., 2005), by alterations in viral loads and protective immunity, and by marked changes in immunopathology (Chen et al., 2001; Selin et al., 1998). This ‘heterologous immunity’ can be a consequence of unanticipated T cell cross-reactivity between different pathogens. Indeed, T cell specificity can be quite degenerate, and cross-reactivity between different viruses is common. For example, human studies have revealed strong T cell cross-reactivity between influenza A virus (IAV) and Epstein-Barr virus (EBV) epitopes (Clute et al., 2005). In some patients, a subset of HLA-A2-restricted EBV-BMLF1 specific T cells is cross-reactive to IAV-M1. A cross-reactive response is also identified between IAV and hepatitis C virus (HCV) epitopes (Urbani et al., 2005). The T cells that are specific to HCV-NS3 epitope can
cross-react with IAV-NA.

A property of heterologous immunity noted both in the humans and mice is that there can be dramatic variation in pathogenesis and in the cross-reactive specificities of the T cell responses between individuals. Mouse studies have shown that such variation can even occur in genetically identical individuals subjected to similar infection histories. In genetically identical LCMV-immune mice challenged with VV, T cell immunodominance and cross-reactivity patterns varied among mice, such that, for example, in some mice VV selectively expanded CD8 T cells cross-reactive with LCMV epitope NP205-212, whereas in other mice there is selective expansion of CD8 T cells specific to GP34-41 or to GP118-125 (Kim et al., 2005). Additionally, T cell adoptive transfer studies indicated that individual LCMV-immune mice had unique patterns of CD8 T cell cross-reactivity to VV, and that the private specificities of the T cell repertoires in individuals regulated the magnitude and the specificities of T cell responses under conditions of heterologous immunity (Kim et al., 2005).

Human diseases involving suspected heterologous immunity, such as EBV-induced acute mononucleosis, HCV-induced hepatitis, and even dengue virus-induced hemorrhagic fever and shock syndrome all present with marked variations in immunopathology between individuals. In this chapter, we tested the hypothesis that variations in immunopathology under conditions of heterologous immunity were, like the specificity of the T cell response, also regulated by the private specificity of the immune repertoire. To do so, we used the mouse model of
heterologous immunity between LCMV and VV.

Naïve C57BL/6 male mice infected IP with $1 \times 10^6$ PFU of VV, strain WR, usually show little or no pathology in visceral fat pads 6 days post infection, while LCMV-immune mice, on infection with VV, develop panniculitis, in the form of acute fatty necrosis (Selin et al., 1998). The levels of panniculitis in LCMV-immune mice were scored at day 6 of VV infection. The variation in levels of panniculitis in LCMV-immune mice ranges from none to very severe levels, as shown in figure 4.1. By using adoptive transfers of splenocytes from LCMV-immune mice into naïve hosts subsequently challenged with VV Fig 1.5, we examined whether the individual differences in pathogenesis of panniculitis were determined by the private specificity of the immune host.

A. **Splenocytes from a single LCMV-immune donor show similar expansions of LCMV-specific CD8 T cell response in adoptively transferred recipients.**

The visceral fat pads that are subject to panniculitis in this model line the peritoneal cavity. To first determine if the response of T cells isolated from infected peripheral tissue is a function of the private specificity of the immune repertoire, as we have shown in the spleen, CFSE-labeled LCMV-immune splenocytes derived from a single donor mouse were transferred into two naïve Ly5.2 congenic
Figure 4.1 Different levels of panniculitis developed in LCMV-immune mice during VV infection. LCMV-immune mice were challenged with $1 \times 10^6$ PFU VV IP. The levels of panniculitis were record at day 6 of VV infection.
recipients. At day 6 of VV infection, the donor peritoneal exudate cell (PEC) responses against LCMV epitopes (NP<sub>396-404</sub>, GP<sub>33-41</sub>, GP<sub>276-286</sub>, NP<sub>205-212</sub> and GP<sub>118-125</sub>) were examined. As shown in figure 4.2, both recipients receiving donor cells from LCMV-immune donor 1 and 3 selectively expanded GP33- and NP205-specific T cells. On the other hand, very little proliferation was observed in GP33-specific CD8 T cells from both recipients receiving cells from LCMV-immune donor 2. Expansions of GP276-specific CD8 T cells were seen in both recipients receiving donor cells from LCMV-immune donor 4. This experiment confirms that the specificity of the memory LCMV-specific T cell response elicited by VV in the periphery is a function of the immune repertoire of the individual immune host.

B. The private specificity of LCMV-immune T cell repertoire determines the levels of immunopathology induced by VV infection.

We have shown previously that the panniculitis in this model of heterologous immunity is mediated by T cells (Selin et al., 1998). Transfers of splenocytes from LCMV-immune mice into naïve mice prior to VV infection could elicit this pathology. Variation in pathology is dramatic among LCMV-immune mice after VV infection (Fig. 4.1), so we tested here whether such variation in pathology could be linked to the private immune repertoire. After transfer of LCMV-immune splenocytes
Figure 4.2 Recipients received splenocytes derived from a single LCMV-immune donor showed similar skewing in LCMV-specific CD8 T cell response after VV challenge. At day 6 of VV infection, LCMV-specific responses of donor cells isolated from PEC were examined by ICCS. Cell division of gated donor CD8 T cells was shown by loss of CFSE, and the specificity of the response was examined by intracellular IFN-γ assay.
derived from a single donor into two naïve recipients, both recipients were challenged with VV. The levels of panniculitis at day 6 of VV infection were recorded. Figure 4.3 shows fat pad pathology from two sets of donor-recipient pairs. The two recipients receiving memory cells from immune donor 1 both showed none or very mild panniculitis, while the fat pads isolated from recipients transferred with cells from donor 2 both showed considerably higher levels of panniculitis. This type of experiment was done four times with 4-5 donors per experiment, and the results are shown in Table 4.1. The two recipients from a given donor had very similar levels of panniculitis after VV challenge. With three exceptions, all paired donor recipients had either identical pathology scores or else pathology scores ± one unit on an eight unit scale (Table 4.1). Two of the exceptions were just two units different. In contrast, the levels of panniculitis in recipients receiving memory cell populations from different LCMV-immune donors varied dramatically and ranged from zero to six (Table 4.1). To compare whether the variation within paired recipients was statistically significantly less than the variation between unpaired recipients, we used intra-class correlation to analyze our data. We found that the two observations of each pair fell into a moderate correlation ($r^2=0.6$, $p=0.002$, Table 4.1). These data indicate that the pathogenesis of panniculitis in LCMV-immune mice during VV infection is also predetermined by the private specificity of the T cell repertoire. One exception was found in experiment 2. In the second pair, host 1 showed mildly moderate level (3) of panniculitis, while the other host had severe (6) panniculitis, which suggested that
Figure 4.3 Recipients received splenocytes derived from a single LCMV-immune donor exhibited similar levels of VV-induced panniculitis. Two recipients transferred with splenocytes derived from a single LCMV-immune donor were infected with VV one day post transfer. At day 6 of infection, the levels of panniculitis were recorded. Representative pictures of two sets of donor-recipient pairs are shown.
Table 4.1 Two hosts receiving splenocytes from one LCMV-immune donor developed similar levels of panniculitis.

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<th>Host 2*</th>
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</tr>
<tr>
<td></td>
<td>1 2</td>
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</tr>
<tr>
<td>Exp II</td>
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</tr>
<tr>
<td></td>
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<td>3 3</td>
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<tr>
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<tr>
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* Hosts 1 and 2 on the same row represent the two mice transferred with splenocytes derived from a single LCMV-immune donor.

# ICC, intra-class correlation.
there might be another uncontrolled factor affecting the levels of panniculitis.

C. Pooled splenocytes from multiple LCMV-immune donors result in similar patterns of heterologous T cell responses and levels of immunopathology in all recipients.

As shown above, similarities in skewing of LCMV-specific CD8 T cell responses and development of panniculitis were observed in paired VV-infected recipients receiving LCMV-memory cells from single donors. We next tested whether pathology scores would be similar in multiple recipients that are transferred of mixed splenocytes from several LCMV-immune mice. Pooled splenocytes from multiple donors were transferred into four or five naïve recipients. At day 6 of VV infection, the LCMV-specific CD8 T cell responses were examined and the levels of panniculitis were recorded. In experiment I, all four recipients receiving pooled LCMV-immune splenocytes generated similar T cell responses, as shown in figure 4.4, where all recipients expanded the NP205-specific T cell population. This result is consistent with the data in figure 4.2, which shows that recipients receiving same donor populations generate similar patterns of skewing of LCMV-specific responses.

Then, we compared the levels of panniculitis that developed in these recipients. We found that most of the recipients developed similar levels of
Figure 4.4 Most of the recipients receiving a same pool of splenocytes from multiple LCMV-immune donors showed similar skewing in LCMV-specific CD8 T cell repertoire. Pooled LCMV-immune splenocytes derived from multiple LCMV-immune donors were transferred into four or five naïve mice, which then were infected with VV. The splenocytes were isolated and examined at day 6 of infection. The pre- and post transfer responses of donor cells are shown. Percentages of epitope-responding CD8 T cells are shown in the top right quadrant. After subtracting the background, the fold increase of the percentage of epitope-specific CD8 T cells during VV infection are indicated in parentheses on the top of the plot. Three independent experiments showed similar results.
panniculitis (Fig 4.5). However, in every experiment, there was one mouse that behaved quite differently from the rest of the recipients. For instance, in experiment 1, one mouse developed a moderately severe level (5) of panniculitis, while others were at very mild level (1). Therefore, our data demonstrated that private specificity of the memory pool played a main effect on determine pathogenesis of panniculitis in LCMV-immune mice during VV infection, though other unknown factors may also be involved.

D. Chapter summary

By using the adoptive transfer technique, for the first time we directly confirm the importance of the private specificity of memory T cell repertoires in determining the pathogenesis of immunopathology during heterologous viral infection. Splenocytes derived from a single LCMV-immune donor induced similar levels of panniculitis in two recipients, while cells from different immune donors induced variations in the levels of panniculitis among recipients. Thus, the levels of panniculitis are determined by the unique features of the memory T cell repertoires. Similar scores of pathology and skewing of the hierarchies of LCMV-specific CD8 T cells were also observed in multiple recipients adoptively transferred with pooled LCMV-immune splenocytes from multiple donors. Taken together, our study indicates that the private specificity is the main contributor in the variations in the
Figure 4.5 Most of the recipients receiving a same pool of splenocytes from multiple LCMV-immune donors showed similar levels of panniculitis. Pooled splenocytes derived from multiple LCMV-immune donors were transferred into four or five naïve mice, which then were infected with VV. At day 6 of infection, the levels of panniculitis were recorded. Graph shows the levels of panniculitis from three independent experiments.
pathogenesis of immunopathology observed in genetically identical individuals during heterologous viral infection.
CHAPTER V:

TNF/LT AND FASL MEDIATE THE PATHOGENESIS OF PANNICULITIS IN LCMV-IMMUNE MICE DURING VV INFECTION

Panniculitis is a group of diseases that involve inflammation of adipose tissue. It is histologically characterized by the infiltration of inflammatory cells and necrosis of fat cells (Requena and Yus, 2001). Erythema nodosum (EN) is the most common form of panniculitis (Requena and Yus, 2008). It presents with painful, tender, erythematous subcutaneous nodules usually 1-5 cm in diameter, and is most commonly located on the anterior portion of the lower legs, arms and soles. The etiology of EN is unknown, but is associated with fungal diseases, infectious mononucleosis, intracellular bacterial and viral infections (Requena and Yus, 2008). It has been reported that EN can occur after vaccination, including with VV for smallpox (Gaertner et al., 2004). Panniculitis may also occur in visceral fat tissue and be extremely debilitating in association with lupus, scleroderma, lymphomas and pancreatitis (Requena and Yus, 2001). Several groups have reported that the utilization of drugs to block the function of TNF improves EN when it presents with autoimmune diseases, such as Crohn’s disease, sarcoldosis or as erythema nodosum leprosum during leprosy (Haley et al., 2004; Kugathasan...
et al., 2003; Roberts et al., 2003; Sheskin, 1980). This is highly suggesting that TNF may be involved in the pathogenesis of EN and other forms of panniculitis.

TNF modulates the immune system at a variety of levels, including inflammation, cell trafficking, activation, differentiation, proliferation, death and development of lymphoid tissue (De et al., 1994; Kim and Teh, 2004; Pasparakis et al., 1997; Singh and Suresh, 2007; Vassalli, 1992; Yu et al., 2003). TNF has a key role in mediating host defense against pathogens including VV, as we have discussed previously (see introduction and Chapter III). Both TNFR1 and TNFR2 have been shown to be required for mediating protection against VV (Chan et al., 2003; Ruby et al., 1997). The protection may be mediated at least partially by killing of the VV infected cells by necrosis. It has been shown that during VV infection, when caspase-8 is inhibited by the viral inhibitor SPI-2 (Shisler and Moss, 2001), signaling through TNFR1 can trigger programmed necrotic death (Holler et al., 2000). It has also been shown that TNFR2 is required to enhance the TNFR1-induced programmed necrosis (Chan et al., 2003). Similar to TNF, FasL and TRAIL, other members of TNF superfamily, can also induce programmed necrosis (Holler et al., 2000). Fas-FasL interactions have been suggested to be involved in some types of autoimmune diseases (Li et al., 2004), as well as fat pathology (Fischer-Posovszky et al., 2006). Fischer-Posovszky et al. found that in patients with acquired lipodystrophy, adipose tissue sections from the regions with ongoing fat loss were positive for Fas. They also found that the co-treatment of
IFN-γ and TNF could sensitize human adipocytes to Fas-mediated apoptosis in vitro. Since the histological analysis of panniculitis shows fat cell necrosis, we hypothesized that TNF, FasL and TRAIL were involved in mediating the enhanced panniculitis in LCMV-immune mice during VV infection. To test our hypothesis, we compared the levels of panniculitis in VV-infected LCMV-immune mice in the presence or absence of TNF, FasL and TRAIL by using either blocking antibodies or genetically deficient mice. This thesis demonstrates that TNF, LT and FasL all participate in enhancing panniculitis in VV infected LCMV-immune mice, but that TRAIL only plays a minor role in this process.

A. TNF and LT are involved in enhancing panniculitis in LCMV-immune mice during acute VV infection.

Prior immunity to LCMV can induce immunopathology manifested by extensive panniculitis, in the form of acute fatty necrosis in visceral fat pads 4-7 days post VV challenge (Selin et al., 1998; Yang et al., 1985). As shown in Figure 1.6A, LCMV-immune mice develop severe and moderate levels of panniculitis on the visceral fat pads after infection with 1.0X10^6 PFU VV IP. In contrast, most of the non-immune mice after infection with VV showed only very mild levels of panniculitis (Fig 1.6A).

As discussed earlier, TNF is a very important inflammatory cytokine, required
for controlling the clearance of variety of pathogens, such as viruses, bacteria and fungi (Schluter and Deckert, 2000). However, It has also been suggested to be involved in tissue damage and human autoimmune diseases (Bolger and Anker, 2000; Moore et al., 1999; Uysal et al., 1997). Thus, we first tested whether the VV-induced panniculitis in LCMV-immune mice was due to the function of TNF and LT by using etanercept to block the activities of both TNF and LT. Two days prior to VV infection, LCMV-immune or naïve C57BL/6 mice were treated with either 100μg etanercept or 100μg control human IgG-Fc. All the mice were infected with 1X10^6 PFU of VV IP. As shown in Fig 5.1A, TNF blockade significantly (p=0.0004) abrogated the enhancement of panniculitis in LCMV-immune mice. At day 6 of VV infection, the LCMV-immune mice that received IgG treatment showed significantly increased panniculitis, as compared to IgG treated naïve mice (p=0.0009). Etanercept treatment efficiently decreased the severity of panniculitis in VV-infected LCMV-immune mice to a similar basal level as seen in TNF blocked naïve mice (p=0.2). The difference in the development of panniculitis between IgG and etanercept treated LCMV-immune mice after VV infection was not due to different rates of viral clearance, as they had similar viral titers in their fat pads and testes at day 6 of VV infection (Chapter III, Fig 3.8).

Private specificity of cross-reactive LCMV memory population induces tremendous variation in the level of acute panniculitis during VV infection as described in the Chapter IV. In order to control for the variable and directly test whether TNF and LT were important for mediating panniculitis induced by
Figure 5.1 Blocking of TNF and LT completely abrogated the increase of VV-induced panniculitis in LCMV-immune mice. A, etanercept (etan)- or IgG-treated naïve and LCMV-immune mice were infected with 1X10^6 PFU VV IP. The levels of panniculitis were recorded at day 6 of infection. B, One pool of LCMV memory splenocytes derived from multiple donors were transferred into 9 or 10 naïve mice. Half of the recipients were treated with etanercept, and the other half received IgG treatment as control. Levels of panniculitis were recorded at day 6 of infection. Pooled results from two independent experiments are shown. (exp I (solid) and exp II (open)). Student’s t test was used for statistical analysis.
cross-reactive LCMV memory cells upon VV infection, we adoptively transferred the same pool of lymphocytes from multiple LCMV-immune donors into a group of WT naïve C57BL/6J mice. Half of these mice then received etanercept treatment, and the rest were treated with IgG as control. The etanercept-treated mice showed significantly lower levels of LCMV-memory cell-induced panniculitis than IgG-treated controls (Fig 5.1B), further suggesting the role of TNF and LT in mediating panniculitis induced by LCMV immunity during acute VV infection.

The fat pads were extracted from IgG- and etanercept-treated VV infected LCMV-immune mice. Examples from both groups are shown in figure 5.2A. The fat pad extracted from the IgG-treated group showed moderate level of panniculitis, while the fat pad from the etanercept-treated mouse had none. Paraffin-embedded fat pads were sectioned at 5μm and stained with hematoxylin and eosin (H&E). As shown in figure 5.2B, the fat pad extracted from IgG-treated mice showed dramatic necrosis of fat cells, and massive infiltration of mononuclear cells into the fat tissue. In contrast, the fat pad isolated from etanercept-treated mice had normal fat tissue structure and showed only mild infiltration of mononuclear cells. Since the LCMV-specific memory CD8 T cells could still be recruited into the fat pads in the etanercept-treated mice (as seen in Chapter III, Fig 3.1), the absence of panniculitis in these mice was not due to the failure of recruitment of LCMV-specific T cells.

To further dissect the role of TNF versus LT on the development of panniculitis,
Figure 5.2 Anti-TNF treated LCMV-immune mice showed little panniculitis and normal fat tissue structure. Etanercept- or IgG-treated LCMV-immune mice were infected with VV. Day 6 of infection, visceral fat pads were isolated. Representative pictures of fat pads are shown in A. B, fat pad sections from IgG treated (a-c) and etanercept-treated (d) LCMV-immune mice infected with VV for 6 days were stained with H&E. (a) Fat pad with moderately severe panniculitis isolated from IgG-treated LCMV-immune mice at day 6 of VV infection showed (b) massive infiltration of lymphocytes (pointed by the arrows) and (c) extensive necrosis of fat cells. (d) Fat pad with no panniculitis from VV-infected etanercept-treated LCMV-immune mice showed normal architecture.
we compared the induction of panniculitis between TNF−/− and WT LCMV-immune mice. It has been shown that TNF is not required for establishing normal immune responses against LCMV infection and LCMV clearance (Calzascia et al., 2007; Klavinskis et al., 1989; Leist and Zinkernagel, 1990; Suresh et al., 2005), so we were able to utilize LCMV immunized TNF−/− mice for our studies. Since naive TNF−/− mice were more susceptible to VV infection (Chapter III), LCMV-immune WT and TNF−/− mice together with their naïve controls were infected with 7.5×10⁵ PFU VV IP. The levels of panniculitis were recorded at day 6 of infection. Consistent with the results from the blocking studies, at day 6 of VV infection the levels of panniculitis were significantly increased in WT LCMV-immune mice, as compared to their naïve controls (p=0.02) (Fig 5.3). TNF−/− LCMV-immune mice showed only a slight increase in the levels of panniculitis compared to TNF−/− non-immune mice (p=0.3). However, when we compared TNF−/− LCMV-immune mice to WT immune mice, we did not find a significant decrease in the levels of panniculitis (p=0.4). These results suggest that TNF may only play a partial role in mediating panniculitis, and that TNF may work together with LT to fully induce the development of panniculitis in LCMV-immune mice during VV infection.
Figure 5.3 TNF alone only played a partial role in mediating panniculitis in LCMV-immune mice during VV infection. LCMV immunized C57BL/6J or TNF−/− mice and their naïve controls were infected with 7.5X10^5 PFU VV IP. At day 6 post infection, significantly increased levels of panniculitis were found in LCMV-immune C57BL/6J mice, but the enhancement of panniculitis was slightly decreased in TNF−/− LCMV-immune mice, as compared to their controls. n=20 per group, error bars represent ±SEM, Student's t test was used for statistical analysis.
B. FasL is required to mediate the pathogenesis of panniculitis in VV-infected LCMV-immune mice.

FasL, another member of TNF superfamily, can also induce apoptosis and programmed necrosis upon engagement with its receptor, Fas (Holler et al., 2000). It has also been shown to be involved with adipocyte apoptosis in patients with lipodystrophy, a syndrome with partial or complete loss of adipose tissue (Fischer-Posovszky et al., 2006). To test whether FasL was also involved in the pathogenesis of panniculitis in our model, we challenged naïve and LCMV-immune gld (FasL-deficient) as well as WT mice with 1.0X10^6 PFU VV IP. As shown in figure 5.4A, both naïve gld mice and LCMV-immune gld mice developed very little panniculitis. LCMV-immune gld mice did not show increased levels of immunopathology after VV infection, compared to gld non-immune mice (p=0.7) (Fig 5.4A). The deficiency in FasL decreased the levels of panniculitis in LCMV-immune mice remarkably (p<0.0001). The absence of panniculitis in VV-infected gld LCMV-immune mice was not due to the failure of recruiting LCMV-specific T cells into infected peripheral tissues. As shown in figure 5.4B, more LCMV-specific CD8 T cells were detected in peritoneal cavity and fat pad in gld mice than in WT mice at day 6 of VV infection. Representative fat pads and histology slides of non-infected mice and VV-infected LCMV-immune WT and gld mice are shown in figure 5.5. The fat pad extracted from a naïve mouse showed healthy intact fat tissue structure (Fig 5.5A). After heterologous VV challenge, the
Figure 5.4 FasL-deficiency abrogated VV-induced panniculitis in LCMV-immune mice completely. LCMV-immune C57BL/6J or FasL-deficient (Gld) mice and their naïve controls were infected with $1 \times 10^6$ PFU VV IP. At day 6 of infection, the levels of panniculitis were recorded (A) and pooled lymphocytes isolated from PEC and fat pads were examined by ICCS (B). B shows a representative out of three independent experiments. Student’s t test was used for statistical analysis.
Figure 5.5. FasL-deficient LCMV-immune mice showed no sign of panniculitis, but massive lymphocyte infiltration in the fat pads during VV infection. Representative pictures and histology slides of fat pads extracted from uninfected naïve controls (A) and day 6 VV-infected WT (B) and gld (C) LCMV-immune mice.
fat pads isolated from WT LCMV-immune mice showed moderately severe levels of panniculitis and massive fat cell necrosis and lymphocyte infiltration (Fig 5.5B). However, most VV-infected gld LCMV-immune mice had no panniculitis (Fig 5.4A), although they still showed large amounts of mononuclear cell infiltrates (Fig 5.5C) and LCMV-specific CD8 T cells into their fat tissue (Fig 5.4B). Thus, FasL is required for the development of panniculitis in LCMV-immune mice during VV infection.

Finally, we tested the role of TRAIL in mediating panniculitis in LCMV-immune mice during VV infection. TRAIL, like TNF and FasL, is also a member of the TNF superfamily. It is important for regulating immune responses and eliminating tumor cells (Aggarwal, 2003). Like TNF and FasL, TRAIL is also able to induce both apoptosis and necrosis (Holler et al., 2000). Death receptor 5 (DR5), the receptor for TRAIL, can be expressed by most cells, including adipocytes (Fischer-Posovszky et al., 2004). Thus, we tested whether the anti-TRAIL treatment would affect the development of panniculitis in LCMV-immune mice after VV challenge. As shown in figure 5.6, there was only a minor effect of TRAIL-blockade on panniculitis. The levels of panniculitis were slightly ($p=0.2$) decreased in mice treated with anti-TRAIL antibody, compared to control mice.

Thus, our data suggest that TNF/LT and FasL are the main effector molecules contributing to the pathogenesis of panniculitis in VV-infected LCMV-immune mice. The low levels of panniculitis generated in gld and etanercept-treated LCMV-immune mice after VV challenge may result from the effect of TRAIL.
Figure 5.6 TRAIL played a minor role in mediating VV-induced panniculitis in LCMV-immune mice. Anti-TRAIL-treated or control WT LCMV-immune mice were infected with 1X10^6 PFU VV IP. Levels of panniculitis were recorded at day 6 of infection. Student’s t test was used for statistical analysis.
C. TNF up-regulates the expression of Fas on adipocytes through TNFR2, but not TNFR1.

The absence of either TNF/LT or FasL completely abolished the incidence of panniculitis in LCMV-immune mice during VV infection, suggesting that TNF/LT and FasL may be working up or down-stream of each other in the same pathway. Many studies have already shown that TNF has many effects on fat cells, such as inducing cytokines, cytokine-induced proteins, enzymes and signaling molecules (Ruan et al., 2002). It has also been shown that a combined treatment of IFN-γ and TNF can up-regulate Fas expression and sensitize human adipocytes for Fas-mediated apoptosis (Fischer-Posovszky et al., 2004; Fischer-Posovszky et al., 2006). To understand how TNF/LT and Fas cooperated with each other to mediate panniculitis, we first tested whether the Fas expression on mouse adipocytes could be regulated by TNF. Fas mRNA levels were measured in the 3T3-L1 adipocyte cell line treated with 5ng/mL TNF for 24 hours in the presence or absence of antagonistic antibodies against TNFR1 or TNFR2. As shown in figure 5.7A, after 24-hour stimulation with TNF, the mRNA of Fas was increased about 60-fold (n=4, paired t test p=0.03). In the presence of antagonistic antibody against TNFR1, the enhancement of Fas transcription only slightly decreased from 60-fold to 50-fold (p=0.1), but the treatment of antagonistic antibody against TNFR2 significantly blocked the up-regulation of Fas mRNA in 3T3-L1 adipocytes. In the presence of TNFR2 antagonistic antibody, the augmentation of Fas mRNA
Figure 5.7 TNF treatments dramatically up-regulated the mRNA expression of Fas, but only increased DR5 mRNA mildly. 3T3-L1 adipocytes 6 days post differentiation induction were treated with 5ng/mL TNF, with the pretreatment of either 1μg/mL of IgG, TNFR1 antagonistic antibody or TNFR2 antagonistic antibody. Total RNA was isolated 24 hours after treatment and analyzed by quantitative real-time PCR for mRNA of Fas (A) and DR5 (B). (n = 4, paired student’s t test was used for statistical analysis).
reduced from 60-fold to 20-fold after 24-hour treatment with TNF, significantly lower than the cells treated with control IgG (n=4, paired t test $p=0.048$). We also tested the up regulation of DR5 by TNF treatment on these adipocytes. The mRNA of DR5 only mildly increased 3-fold after 24-hour TNF treatment, and blocking of either TNFR1 or TNFR2 alone could not affect this enhancement (Fig 5.7B).

TNF and LT exert their biological functions through two receptors, TNFR1 and TNFR2. The fact that the intracellular sequences of TNFR1 and TNFR2 are largely unrelated with almost no homology between each other suggests that TNFR1 and TNFR2 may induce different signaling pathways in response to TNF signaling. Our data shown in figure 5.7A suggests that it is TNFR2, but not TNFR1 that is responsible for regulating Fas expression on adipocytes. To test whether TNFR2 was involved in mediating panniculitis in LCMV-immune mice during VV infection, we adoptively transferred WT LCMV memory splenocytes derived from a single donor into one WT naïve host and one TNFR$^{-/-}$ naïve host. Both recipients were then infected with $1.0 \times 10^6$ PFU VV IP one day post transfer. As shown in chapter IV, the levels of VV-induced panniculitis in LCMV-immune mice was determined by the LCMV-specific memory population, as two C57BL/6J hosts receiving the same LCMV memory population generated similar levels of panniculitis after VV infection (Fig 4.3). If the tested TNF receptor was involved in mediating this effect, the TNFR$^{-/-}$ host would show decreased level of disease compared to the WT control who received the same donor cells. After VV infection, TNFR2$^{-/-}$ mice showed significantly (10 pairs, paired t test $p=0.003$) diminished pathology, as
compared to their WT controls (Fig 5.8A). The decrease in levels of panniculitis in TNFR2−/− mice was not due to different levels of viral clearance, as both KO mice and WT mice showed similar VV loads in their fat pads and testes (Fig 5.8B). The absence of panniculitis was also not because of the failure of donor T cell survival in TNFR2−/− mice, since the KO recipients showed comparable numbers of donor LCMV-specific CD8 T cells accumulated in peritoneal cavity and fat pads as their WT controls (Fig 5.9). To test whether the expression of TNFR2 on the memory T cells would also be important for inducing panniculitis, LCMV memory cells derived from TNFR2−/− or WT LCMV-immune donors were transferred into WT naïve hosts. At day 6 of infection, the recipients receiving TNFR2−/− memory cells generated similar levels of panniculitis (Fig 5.10A) and had comparable VV loads (Fig 5.10B) as their controls receiving WT memory cells. Therefore, the TNFR2 expression on the recipient cells, most likely on the adipocytes, is important for the enhancement of VV-induced immunopathology in LCMV-immune mice.

In contrast to TNFR2−/− mice, TNFR1−/− recipients and WT control mice receiving WT LCMV memory cells derived from a single immune donor had comparable levels of pathology (10 pairs, paired t test p=0.6) (Fig. 5.11A). However, we found TNFR1−/− recipients had higher levels of virus titers in their fat pads and testes than their controls, as shown in figure 5.11B. This may affect the induction of panniculitis in TNFR1−/− mice, as the death of fat cells may result from very high viral loads. Thus it was difficult to conclude whether TNFR1 played a role in mediating LCMV-specific memory cell-induced panniculitis during VV infection.
Figure 5.8 TNFR2 was required for LCMV immunity mediated panniculitis during VV infection. Splenocytes from one LCMV-immune Ly5.1 mouse were equally adoptively transferred into one naïve WT host and one TNFR2-/- host. Both animals were then challenged with $1.0 \times 10^6$ PFU VV IP. A, at day 6 of infection, the levels of panniculitis were recorded and compared between TNFR2-/- and WT controls. B shows a representative result of virus titers of fat pad and testis. Open bars and black bars represent TNFR2-/- hosts and WT controls, respectively. n=5 per group, error bars represent ±SEM, paired student’s t test was used for statistical analysis.
Figure 5.9 TNFR2⁻⁻ recipients showed comparable numbers of donor LCMV-specific CD8 T cells as WT controls. Pooled PEC and lymphocytes isolated from fat pads (FP) at day 6 of VV infection were examined by ICCS. A representative out of three independent experiments is shown.
Figure 5.10 TNFR2 expression on the surface of LCMV-specific memory T cells was not required for mediating VV-induced panniculitis. Splenocytes derived from LCMV-immune TNFR2<sup>−/−</sup> and WT donors were transferred into naïve recipients, respectively. Recipients were then infected with 1X10<sup>6</sup> PFU VV IP. At day 6 of infection, levels of panniculitis (A) and VV loads (B) were examined. Student’s t test was used for statistical analysis.
Figure 5.11 TNFR1<sup>−/−</sup> recipients showed similar levels of panniculitis but higher viral loads than WT control recipients after regular dose VV infection. Splenocytes derived from a single WT LCMV-immune donor were transferred into one WT and one TNFR1<sup>−/−</sup> naive recipient. Both recipients were then infected with 1X10<sup>6</sup> PFU VV IP. At day 6 of infection, levels of panniculitis (A) and VV loads (B) were examined. Paired student’s t test was used for statistical analysis.
To test whether the pathology generated in TNFR1−/− mice would be diminished if they could control the viral loads to a comparable level to their WT controls, we infected TNFR1−/− recipients with a lower dose of VV. One day after transfer of LCMV memory cells, TNFR1−/− mice were infected with 6.0×10^5 PFU VV, while WT controls were infected with 1.0×10^6 PFU VV. At day 6 of infection, the levels of panniculitis were recorded, and the virus titers of fat pads and testes were examined. With a lower infection dose, TNFR1−/− mice could control the viral loads to the same levels as their controls (Fig 5.12B). However, they still showed similar, if not increased levels of panniculitis as the WT controls (Fig 5.12A, 5 pairs, paired t test p=0.1). A similar experiment was done with the TNFR1−/− recipients receiving normal dose (1.0×10^6 PFU) of VV, and the WT control infected with a higher dose of VV (1.5×10^6 PFU). TNFR1−/− mice still developed comparable levels of panniculitis as their WT controls did (data not shown).

In summary, these results suggest that TNF and LT signal through TNFR2 to up-regulate the expression of Fas on the adipocytes. Although TNFR1 can mediate programmed necrosis (Holler et al., 2000), it does not seem to be involved in the pathogenesis of panniculitis in this model.
Figure 5.12 TNFR1 did not play a role in LCMV immunity mediated panniculitis during VV infection. Splenocytes derived from a single WT LCMV-immune donor were transferred into one WT and one TNFR1⁻/⁻ naïve recipient. TNFR1⁻/⁻ and WT recipients were then infected with 6\times10^5 and 1\times10^6 PFU VV IP, respectively. At day 6 of infection, levels of panniculitis (A) and VV loads (B) were examined. Paired student’s t test was used for statistical analysis.
D. VV infected LCMV-immune mice show increased production of TNF by cross-reactive CD8 T cells.

As shown by a previous study, the fat pads of VV infected LCMV-immune mice are infiltrated with activated CD8 T cells specific for LCMV (Selin et al., 1998) (Chapter III, Fig 3.1). Adoptive transfer studies showed that the enhanced immunopathology was mediated by LCMV memory T cells (Selin et al., 1998). Transfer of either CD4 T cells or CD8 T cells from LCMV-immune mice was sufficient to induce panniculitis in recipients during acute VV infection (Selin, unpublished data), and the level of panniculitis was determined by the memory population of the host (Chapter IV). Hence, the cross-reactive LCMV-specific T cells play a crucial role in initiating development of panniculitis. Since TNF also plays a very important role in mediating this disease as we have shown above, we tested whether the production of TNF was different in cross-reactive LCMV-specific T cells than primary activated VV-specific T cells. To do so, we analyzed the responses against two VV epitopes, VV-a11r198 and e7r130. VV-a11r198 is able to elicit a VV-specific T cell response as well as a cross-reactive LCMV-specific T cell response. However, e7r130 can only induce a VV-specific response, but no cross-reactive response from LCMV-specific memory CD8 T cells. We used these two peptides to stimulate the lymphocytes isolated from fat pads of VV-infected LCMV-immune and non-immune mice at day 6 of VV infection. After a 5-hour in vitro stimulation, epitope-specific cells were gated by IFN-γ
production, as shown in figure 5.13. Then IFN-γ⁹+ cells were analyzed for the production of TNF. VV-a11r-specific CD8 T cells from LCMV-immune mice produced more TNF per cell, compared to a11r-specific cells from non-immune mice (Fig. 5.13A and Table 5.1). The increased TNF production was not observed when fat pad lymphocytes were stimulated with the non-cross-reactive epitope VV-e7r₁₃₀ (Fig. 5.13B and Table 5.1). Eight independent experiments are shown in table 5.1. Although the MFI of TNF varied between experiments, the VV-a11r-specific CD8 T cells always had higher TNF production in LCMV-immune mice than in non-immune mice in each experiment (paired t test, p=0.02). These data suggest that in the fat pads the cross-reactive LCMV-specific CD8 T cells produce more TNF than the primary VV effector CD8 T cells upon activation by VV epitope.

However, higher TNF production by the VV-a11r-specific CD8 T cells from LCMV-immune mice was not found in PEC or splenocytes (data now shown). Increased levels of TNF were also not evident in the serum of LCMV-immune mice during VV infection, as compare to non-immune mice (data not shown). However, TNF exists both as soluble and membrane-bound form. The assay that we used to examine the serum level of TNF could not detect the membrane-bound form of TNF. Also the absence of increased TNF in the serum might be explained by the fact that the production of TNF was tightly controlled and localized by the extensive
Figure 5.13 Cross-reactive memory CD8 T cells produced higher levels of TNF, compared to primary effector CD8 T cells. Pooled lymphocytes isolated from the fat pads of naïve (dotted line) and LCMV-immune C57BL/6J (solid line) mice at day 6 of VV infection were stimulated with (A) the cross-reactive epitope a11r and (B) the non-cross-reactive epitope e7r for 5 hours. Cells were then stained for CD44, CD8 and for intracellular IFN-γ and TNF. TNF production was analyzed on CD8⁺, CD44⁺ and IFN-γ⁺ T cells.
Table 5.1 Cross-reactive CD8 T cells produced higher level of TNF than non-cross-reactive CD8 T cells at per cell basis (MFI)

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Lymphocytes isolated from the fat pads of naïve and LCMV immune C57BL/6J mice at day 6 of VV infection were stimulated with VV peptides for 5 hours, and then stained for cell CD44, CD8 and for intracellular IFN-γ and TNF. TNF production (MFI) was analyzed on CD8⁺, CD44⁺ and IFN-γ⁺ T cells.
amount of TNFR within the fat tissue (Mohamed-Ali et al., 1999).

Normally, during VV infection, naïve C57BL/6J mice establish a dominant VV-b8r response and sub-dominant VV-e7r response. The response against VV-a11r is very limited (Cornberg et al., 2007). However, in a LCMV-immune mouse, during VV infection the cross-reactive LCMV-specific memory T cells can be activated by the VV epitope, and then selectively expand and be recruited into the peritoneal cavity and fat pads, sometimes resulting in an increased a11r response (Cornberg et al., 2007; Kim et al., 2005) (Cornberg et al, manuscript submitted). These data suggested that LCMV-immune mice may have higher levels of TNF production in their fat pads at both per cell basis and percentage basis than non-immune mice.

E. Chapter summary

The data shown in this chapter demonstrate the role that TNF/LT and FasL play in mediating panniculitis in LCMV-immune mice during VV infection. Both TNF/LT and FasL were required to increase the levels of VV-induced panniculitis in LCMV-immune mice, since blocking of TNF/LT by etanercept treatment and FasL-deficiency completely abrogated the enhancement. On the other hand, TRAIL only played a minor role in mediating panniculitis, as the augmentation of panniculitis was just partially decreased by the treatment of TRAIL blocking
antibody. By transferring LCMV memory cells into WT and TNFNR\(^{-/-}\) mice, we found that TNFR2, but not TNFR1 was involved in mediating panniculitis. Transferring TNFR2\(^{-/-}\) LCMV memory cells did not show any effect on the pathogenesis of panniculitis, suggesting that the target of TNF/LT was in the recipient cells, but not the cross-reactive memory T cells. We also showed that Fas mRNA was significantly increased in the mouse adipocyte cell line, 3T3-L1 after TNF treatment. Consistent with the results from in vivo experiments, the up-regulation of Fas mRNA in the adipocytes was mainly induced by TNFR2 signaling. We also found that the cross-reactive epitope a11r-specific CD8 T cells isolated from LCMV-immune mice produced higher levels of TNF than cells isolated from non-immune controls, at single cell basis. This phenomenon was not observed when cells were stimulated with VV-e7r, which can not elicit cross-reactivity.

Thus, we conclude that in LCMV-immune mice during VV infection, the cross-reactive CD8 T cells produce higher amounts of TNF in the fat pad. Other types of cells, such as macrophages may also produce more TNF in response to IFN-\(\gamma\) and TNF produced by CD8 T cells. Locally produced TNF may stimulate the adipocytes to up-regulate the expression of Fas on their surface. Then Fas may directly induce the necrosis of the fat cells and the formation of panniculitis, when it interacts with FasL expressed on activated VV-specific and cross-reactive LCMV-specific T cells.
CHAPTER VI: DISCUSSION

Based on the effects that TNF has on regulating immune responses, we hypothesized that TNF was required for mediating heterologous protection against VV in LCMV-immune mice during VV infection. Our data shown in this thesis indicate that TNF does not play a role in mediating heterologous protection in VV-infected LCMV-immune mice. Although TNF plays a role in limiting VV infection in immunologically naïve mice, TNF-deficient (TNF-/- or etanercept-treated) LCMV-immune mice are as resistant to VV infection as WT LCMV-immune mice (Fig 3.7-3.10). Therefore, prior immunity to LCMV can completely abrogate the role of TNF for VV clearance.

Since TNF is implicated in many human autoimmune diseases, including human erythema nodosum (EN), we hypothesized that TNF could contribute to the pathogenesis of immunopathology, i.e. panniculitis in VV-infected LCMV-immune mice. The data shown in chapter V support our hypothesis, as we found the VV-induced panniculitis was significantly diminished in TNF-deficient LCMV-immune mice. By testing TNFR-/- mice, we showed that TNFR2, but not TNFR1 was involved in mediating VV-induced panniculitis, as the levels of panniculitis were significantly decreased in TNFR2-/- mice (Fig 5.8). Furthermore, we found that signaling through TNFR2 could dramatically increase Fas mRNA expression on mouse 3T3-L1 adipocytes, and FasL-deficiency led to an
elimination of this immunopathology in VV-infected LCMV-immune mice. These data establish that TNF is involved in the pathogenesis of panniculitis something which had been suspected in human disease. Our results reveal a pathway that TNF goes through TNFR2 and Fas to induce enhanced fat tissue damage, which may be useful for the treatment of human EN or other types of panniculitis, for which there is still no definitive treatment.

The severity of panniculitis is dramatically different among VV-infected LCMV-immune mice. It has been shown by our group that the VV-induced panniculitis in LCMV-immune mice is mediated by cross-reactive LCMV-specific T cells (Selin et al., 1998), and the cross-reactive responses are determined by the private specificity of the immune host (Kim et al., 2005). We hypothesized that the variation in the levels of panniculitis in VV-infected LCMV-immune mice is determined by the private specificity of the memory T cell repertoire. By adoptively transferring splenocytes derived from a single LCMV-immune donor into two recipients, we were able to show that the levels of immunopathology were similar in recipients receiving the same donor cells. However, there were variations in recipients receiving different donor cells (Fig 4.3 and Table 4.1). These results support our hypothesis, and implicate that the variation in disease outcomes following human viral infection may be affected by the unique private specificity of the immune memory T cell pool of each individual. Furthermore, we showed that recipients receiving a pool of memory T cells with more variety in the TCR repertoire tended to develop little pathology, implicating the importance of a
A. Private specificity of memory TCR repertoire determines individual variations in the pathology.

Mice receiving splenocytes derived from a single LCMV-immune donor showed significantly less variation in levels of panniculitis after VV challenge than mice that received donor splenocytes from different donors (Fig 4.3 and Table 4.1). These results indicate that the private specificity of an individual’s unique T cell repertoire is the major determinant of the highly variable disease course observed in genetically identical LCMV-immune mice during VV infection. It may also provide an explanation for the variations in human disease during viral infection, in addition to the effect of genetic differences, physiologic conditions and others. Since humans are not immunologically naïve, the results of a viral infection can be greatly affected by heterologous immunity. The disease course can be ultimately variable among individuals, especially in teenagers and young adults, who have a longer history of infections and potentially a more complex memory T cell pool than young children, and are more likely to be affected by heterologous immunity. Some viruses, such as Epstein-Barr virus (EBV), can lead to more severe symptoms in teenagers and young adults than in young children, suggesting the influences of heterologous immunity in the older group. EBV infects over 90% of
the U.S. population. Normally, the primary infection of EBV during childhood is asymptomatic. However, primary infection during adolescence frequently presents as infectious mononucleosis (IM), a T cell mediated pathology. Older adults acutely infected with EBV may have even worse symptoms than young adults. They commonly have complications associated with IM, such as hepatomegaly, jaundice and fulminant hepatitis (Auwaerter, 1999). Similar to VV, EBV is also a large DNA virus and potentially encodes multiple T cell epitopes that may elicit cross-reactive T cell responses. As shown previously by our group, a subset of T cells with specificity for a HLA-A2.1-restricted immunodominant EBV epitope, BMLF-1_{280-288}, can cross-react with influenza A virus (IAV) epitope M1_{58-66} (Clute et al., 2005). Though IM patients frequently show an increase in the number of IAV M1-specific T cells in their blood, this identified cross-reactive response between EBV and IAV was only found in some but not all IM patients (Clute et al., 2005), which might be a reflection of the uniqueness of private specificities of these patients. More cross-reactive T cell responses have been identified by our group and a correlation of these cross-reactive responses with disease severity has also been found (Aslan, unpublished data).

Another viral disease that presents with high individual variations in symptoms is dengue virus. Dengue is a mosquito-born virus that affects more than 100 countries, and billions people are at risk of dengue infection. It causes millions to be ill and thousands of deaths every year (Gibbons and Vaughn, 2002; Guzman and Kouri, 2002). The result of dengue virus infection can range from
asymptomatic infection, dengue fever (DF), to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The pathology induced during dengue infection is also age related, with none or little symptoms observed in young children and more severe diseases developed among older children and adults (Gibbons and Vaughn, 2002). Though dengue is a small RNA virus, it has four distinct but closely related serotypes. Infection with one serotype provides lifelong protection against that serotype, but has heterologous immunities against other serotypes (Gibbons and Vaughn, 2002). It is believed that a majority of the cases of DHF/DSS is associated with secondary infection by a different serotype of dengue (Guzman et al., 2000; Sangkawibha et al., 1984). It may be explained by the presence of antibodies that are specifically against the first serotype, cross-reactive to the second serotype. Upon binding to the targets, these antibodies can not neutralize the virus, but enhance the entry of virus into cells (Vaughn et al., 2000). However, a secondary infection is neither sufficient nor necessary for a dengue patient to generate severe disease. Since only a small number (2-4%) of patients with secondary infection experience DHF/DSS (Guzman and Kouri, 2002), and not all DHF/DSS cases are resulted from secondary infections (Bravo et al., 1987; Wichmann et al., 2004). Cross-reactive T cell responses can also contribute to increased disease severity. It has been shown that the selective expansion of cross-reactive T cells with low binding affinity for the current infection from the unique memory T cell pool of an individual results in inefficient immune response (Mongkolsapaya et al., 2003) and
progression to severe forms of disease (Fink et al., 2006).

Similar to dengue infections, hepatitis C virus (HCV) infections can also induce extremely different outcomes between individuals. This small RNA virus infects more than a hundred million people worldwide. Though the acute infection is frequently asymptomatic, about one third of adults acutely infected show clinical symptoms, which vary from mild to severe illness (Urbani et al., 2005). The outcomes of HCV infection are also varied from viral clearance to persistent infections (Lloyd et al., 2007). Viral-specific CD8 T cell responses are crucial for controlling HCV replication. One study suggest that HCV-infected patients with more severe pathologies may be associated with a narrowly focused cross-reactive response against HCV antigens (Urbani et al., 2005). In this study, the two patients with most severe liver pathology showed a cross-reactive response between the HLA-A2.1-restricted HCV epitope NS3_{1073-1081} and the IAV epitope NA_{231-239}, which is absent in other patients with only mild disease (Urbani et al., 2005).

Collectively, these data and our findings suggest that the pathogenesis of immunopathology during viral infections can be greatly influenced by the distinctive feature of the memory T cell repertoire of the host. The differences in the memory TCR repertoire among individuals determine the presence of variations in disease courses and outcomes of infection.
B. Divergent TCR repertoire is important for efficient host defense.

The effects of heterologous immunity on the memory T cell repertoire have been extensively studied in mice sequentially infected with LCMV and PV (Cornberg et al., 2006). The MHC class I K$^b$-restricted NP$_{205-212}$ epitopes encoded by LCMV and PV have six out of eight amino acids in common, and induce cross-reactive responses between these two viruses. As mentioned above, LCMV infection of naïve C57BL/6J mice generates a common hierarchy of LCMV-specific responses, as the response is co-dominated by NP$_{396}$ and GP$_{33/34}$ responses, while GP$_{276}$, NP$_{205}$ and GP$_{118}$ responses are subdominant (see Introduction). Infection with LCMV also results in a dominant usage of the V$\beta$16 family by NP$_{205}$-specific CD8 T cells, which show a broad clonotype usage. However, as shown in figure 6.1, heterologous challenge of LCMV-immune mice with PV leads to an enhanced expansion of the usually subdominant cross-reactive NP$_{205}$-specific population. This is associated with oligoclonal expansion of other V$\beta$ families and a narrowing down of the clonotypes used within these families (Cornberg et al., 2006; Welsh et al., 2006). These changes are resulting from the selective expansion of the memory T cells that are cross-reactive to PV and the attrition of the non-cross-reactive ones (Brehm et al., 2002; Cornberg et al., 2006; Selin et al., 1999). Skewing of the TCR repertoire during heterologous immunity was also reported in human EBV infections (Clute et al., 2005). The cross-reactive
Figure 6.1 Modulation of the T-cell repertoire during heterologous viral infection. Different dots represent T-cells with different specificities. A naive immune system challenged with either LCMV or PV shows expansions of virus-specific T cells. When the infection is resolved, some of these cells undergo apoptosis. If an immune system that has been conditioned by one virus infection (LCMV) is exposed to another virus (PV), T-cell populations that are cross-reactive with the two viruses (red outline) will expand preferentially and dominate the response, while the memory T cells that are specific for the first virus are reduced in number, which results in a skewing in the hierarchy of the T cell response against the first virus. This figure was taken from Welsh and Selin 2002 Nat Rev Immunol 2: 417-426.
response between IAV-M1 and EBV-BMLF1 induced a skewed hierarchy of Jβ usage by IAV-M1-specific CD8 T cells. In healthy IAV immune donors, the Jβ usage of the Vb17⁺ M1-specific CD8 T cells was dominated by Jβ2.7 (55-62%) followed by Jβ2.3 (10-20%), and Jβ2.1, 2.5, 1.1 and 1.2 were present at lower frequencies (Naumov et al., unpublished observation). However, in Clute’s study the two patients with EBV-associated IM showed substantial changes in the hierarchies of Jβ usage by M1-specific Vβ17⁺ CD8 T cells. In one patient, Jβ1.2 family was predominantly used by about 50% of these cells. An increased Jβ2.3 family (30%) and decreased Jβ2.7 family (10%) usage was seen in another patient (Clute et al., 2005). Thus, sequential infections tend to narrow down the TCR repertoire by selectively expanding a small subset of cross-reactive T cells. However, the dramatically narrowed TCR repertoire may be not able to provide efficient protection against infections the host will experience in the future.

A diverse T cell response is helpful for the host to provide better protection than a narrowly focused T cell response. Such as during HCV infection, vigorous CD8 T cell responses specific to multiple HCV epitopes are associated with viral clearance. However, the HCV-specific CD8 T cell responses found in chronically infected patients and animals are usually weak and narrowly focused (Lloyd et al., 2007). One explanation for the inefficient protection mediated by narrowly focused T cell response is the escape mutation of the virus (Cornberg et al., 2006). When PV-immune mice were infected with 2X10⁵ PFU of LCMV (clone 13), they
established persistent LCMV infection and a narrowed NP_{205}-specific CD8 T cell repertoire. Eight months after LCMV clone 13 inoculation, a variant with a mutation from valine to alanine in the third position of the LCMV-specific NP_{205} epitope was found (Cornberg et al., 2006). Although this mutant peptide could bind to MHC-K^{b} molecule and present to the T cells, it was less efficient than WT NP_{205} epitope in inducing IFN-γ production by CD8 T cells isolated from LCMV-infected naïve or PV-immune mice (Cornberg et al., 2006).

The scope of the T cell repertoire may greatly affect the result of an infection. As shown in Chapter IV, when mice were transferred with pooled splenocytes from multiple LCMV-immune donors, only 20% of the mice (3 out of 14) showed moderate to severe levels of panniculitis. However, when mice received splenocytes derived from one donor, about 40% (15 out of 38) of the mice showed moderate to severe levels of panniculitis after VV infection (Fig 4.3B, Table 4.1). Since every LCMV-immune mouse has a distinct memory T cell repertoire, the pooled splenocytes derived from multiple LCMV-immune donors have a more diverse TCR repertoire than splenocytes from a single donor. A recipient that obtained a diverse TCR repertoire will have a greater chance of eliciting a beneficial cross-reactive T cell response.

Highly skewed or focused T cell responses can be dangerous. This is because they do not provide efficient immune surveillance, and may not generate efficient cross-reactive T cell responses, which may mediate severe tissue
damage. Our results have strong application for vaccine designs. For example, an attenuated vaccine may induce a broad T cell response to multiple epitopes, which will provide better protection against this pathogen. Vaccines that focus on a single T cell epitope of a pathogen might be risky and should be avoided, because the resulting memory T cell response would not be able to clear an unrelated pathogen efficiently and result in severe tissue damage (Welsh and Fujinami, 2007).

C. The role of TNF in mediating protective immunity and immunopathology

Secreting cytokines is one of the most important effector functions CD8 T cells play to control viral infections, such as LCMV (Klavinskis et al., 1989), VV (Chan et al., 2003; Lidbury et al., 1995; Ruby et al., 1997; Sambhi et al., 1991), hepatitis B virus (HBV) (Kasahara et al., 2003), herpes simplex virus (Bouley et al., 1995), respiratory syncytial virus (RSV) (Ostler et al., 2002) and EV (Ruby et al., 1997). Exposure to cytokines can directly reduce viral replication, increase MHC class I expression and up-regulate cellular genes known to increase resistance to viral infection (Slifka and Whitton, 2000b). However, the production of inflammatory cytokines in response to infection may result in development of immunopathology.

As shown in Chapter III, neither IFN-γ nor TNF/LT was important for the selective expansion and recruitment of cross-reactive LCMV-specific CD8 T cells
in VV-infected LCMV-immune mice (Fig 3.1, 3.3 and 3.4). However, previous studies by our group have shown that IFN-\(\gamma\) produced by cross-reactive LCMV-specific memory T cells is required for mediating both heterologous protective immunity against VV and VV-induced immunopathology (Chen et al., 2001; Selin et al., 1998). The absence of TNF and LT did not affect the heterologous protection against VV provided by LCMV immunity; although it substantially diminished the levels of immunopathology (Fig 5.1). This suggests that TNF and LT signaling plays a role in pathogenesis of panniculitis in LCMV-immune mice challenged with VV.

This is supported by data from a mouse RSV model. When mice were infected with RSV, they could clear virus, but developed severe lung injury. IFN-\(\gamma\) produced by virus-specific CD8 T cells was required for both viral clearance and immunopathology (Ostler et al., 2002). On the other hand, TNF played a major effect on mediating immunopathology associated with RSV and influenza, but only a minor role in antiviral activity (Hussell et al., 2001; Ostler et al., 2002; Rutigliano and Graham, 2004).

Since TNF is important in mediating immunopathology during multiple viral infections, it is important to understand its source. In 2006, Liu et al. reported that the administration of cognate synthetic peptides to mice, that were either acutely infected or long-term immune, could cause intestinal pathology. The pathogenic effects of the peptide vaccine were dependent on the presence of epitope-specific
CD8 T cells, because pathology was not seen in CD8 T cell-depleted mice or naïve mice after injection of peptide. Since TNFR1⁻/⁻ mice did not show signs of illness after peptide injection, TNF was also suggested to be involved in the pathogenesis of this pathology. When the authors further examined the TNF producing cells, they found that most of the TNF⁺ cells were also CD8⁺. Therefore, the data of this study suggest that the peptide-induced symptom is dependent on TNF production by the large pre-existing pool of epitope-specific CD8 T cells (Liu et al., 2006). Consistent with Liu’s study, we found the presence of cross-reactive LCMV-specific memory T cells and TNF were also responsible for the pathogenesis of panniculitis in VV-infected LCMV-immune mice. These cross-reactive LCMV-specific memory T cells are present at higher frequency than the naïve VV-specific precursors. Memory cells can also respond to antigen more quickly and stronger than naïve cells, and may cause a highly inflammatory environment. In such a situation, a large amount of TNF might be produced by activated T cells as well as macrophages. By comparing TNF production by antigen-specific CD8 T cells, we found that VV-a11r-specific CD8 T cells isolated from the fat pads of VV-infected LCMV-immune mice produced higher levels of TNF than cells isolated from non-immune mice. However, there was no difference in TNF production by VV-e7r-specific CD8 T cells isolated from the fat pads of VV-infected LCMV-immune and non-immune mice (Fig 5.13 and Table 5.1). The difference between these two VV epitopes is that VV-a11r can elicit cross-reactive response from LCMV-specific memory CD8 T cells, but VV-e7r can not. From this,
we concluded that the reactivated cross-reactive LCMV-specific CD8 memory T cells produced higher amounts of TNF than VV-specific CD8 T cells. This is consistent with the data shown by Slifka et al. that memory CD8 T cells produced more TNF than primary effector CD8 T cells after direct ex vivo stimulation with peptide (Slifka and Whitton, 2000a). Collectively, our data indicate that TNF has no effect on controlling VV infection during heterologous challenge in LCMV-immune mice. However, the increased expression of TNF in the fat pads is involved in immunopathology.

Although TNF can interact with two receptors, most of the biologic functions of TNF were ascribed to TNFR1. Surprisingly, in this thesis research we found that it was TNFR2, independent of TNFR1 that was involved in the pathogenesis of panniculitis in LCMV-immune mice during VV infection (Fig 5.8). Our data are consistent with Douni and Kollias’ finding that TNFR2 plays a critical role in multi-organ inflammation (Douni and Kollias, 1998). They found that at levels relevant to human disease, the production of human TNFR2 (hTNFR2) in transgenic mice could trigger the inflammation in multiple organs, resulting in shorten lifespan. This process was TNFR1-independent, as TNFR1−/− mice expressing hTNFR2 at high level had a shortened lifespan similar to WT mice expressing hTNFR2. An association of TNFR2 and human autoimmune disease has also been suggested. It has been reported that TNFR2 196R, a more effective polymorphism of TNFR2, is associated with familial rheumatoid arthritis (Dieude et al., 2002) and systemic lupus erythematosus (Komata et al., 1999). Collectively,
these data and our results suggest TNFR2 may have a unique role in causing inflammation and tissue damage.

**D. Pathogenesis of panniculitis in LCMV-immune mice during VV infection and its implication for human disease.**

Considering to the importance of TNF in regulating immune responses and its association with human autoimmune disease, we hypothesized that TNF would be required for mediating heterologous protection against VV and immunopathology in VV-infected LCMV-immune mice. Our data shown in this thesis confirm our hypothesis and demonstrate a pathway that TNF goes through to mediate VV-induced fat tissue damage in LCMV-immune mice (Fig 6.2). During VV infection, antigen presenting cells (APC) present VV epitopes to T cells. In LCMV-immune mice, the LCMV-specific memory T cells that are cross-reactive to VV-epitopes re-activate and accumulate in the sites of infection, such as fat pads. These cells then rapidly start to produce high levels of IFN-γ (Chen et al., 2001) and TNF (Fig 5.13). IFN-γ production not only eliminates VV infection efficiently, but also increases inflammation by activating the APC to produce more TNF. TNF and LT signal through TNFR2 on adipocytes and induce the up-regulation of Fas expression. When Fas engages its ligand on activated VV-specific T cells and cross-reactive LCMV-specific T cells, it induces the necrosis of fat cells and causes
Figure 6.2 Model of pathogenesis of panniculitis in LCMV-immune mice heterologously challenged with VV. The cross-reactive LCMV-specific T cells recognize VV epitopes presented on APC and get activated, resulting in the production of IFN-γ and TNF. The release of IFN-γ mediates the rapid clearance of VV and further activated APC to produce TNF. TNF signals through TNFR2 on the adipocytes to up-regulate the expression of Fas, which induces necrosis upon encounters with FasL expressed on activated T cells.
panniculitis. In the whole process, TNF does not play a role in clearing VV from LCMV-immune mice, since the clearance of VV in etanercept-treated LCMV-immune mice is as efficient as control LCMV-immune mice.

Our finding implicates one possible mechanism for the pathogenesis of panniculitis associated with human infections. During infection or vaccination, the reactivated pre-existing cross-reactive memory T cells may mediate a strong inflammatory response with increased cytokine production. These cytokines then go through autocrine as well as paracrine signaling to induce more cytokine production. For instance, IFN-\(\gamma\) activates macrophages to produce more TNF, and TNF works on NK cells and cross-reactive T cells to increase IFN-\(\gamma\) production. As mentioned earlier, TNF can induce inflammatory cytokine production by adipocytes, including IFN-\(\beta\) and IL-6 and leptin (Ruan et al., 2002). In turn, leptin is also known to positively regulate the production of TNF, IL-6 and IL-12 by macrophages (Matarese et al., 2007). Thus, as a consequence of these feedback loops, the host will experience a cytokine storm. During this uncontrollable inflammatory condition, Fas is up-regulated on the surface of adipocytes. Upon interaction of Fas with FasL, which is expressed on activated T cells in the adipose tissue, necrosis of adipocytes occurs.
E. Safety of anti-TNF treatment in humans

The inappropriate production of TNF has been reported to be involved in many types of human autoimmune diseases, such as type II diabetes mellitus, rheumatoid arthritis (RA) and chronic heart failure (Aggarwal, 2003). An association of TNF with human EN has been suggested since anti-TNF treatment efficiently improves the symptoms of EN (see Introduction). In this thesis, we showed that the VV-induced panniculitis was completely abolished by treatment with etanercept (Fig 5.1 and 5.2). Additionally, the levels of panniculitis were significantly reduced in TNFR2−/− hosts, compared to the WT controls (Fig 5.8). These data directly show that TNF and LT are involved in the pathogenesis of panniculitis in LCMV-immune mice during VV infection. According to our data, we suggest that anti-TNF therapy may be considered as a treatment for human EN or other types of panniculitis, for which at the present time there is no definitive therapy.

Worldwide about half million patients have received anti-TNF treatment. It has been shown that anti-TNF therapies are very effective for treatment of Crohn's disease (Present et al., 1999; Suenaert et al., 2002) and RA (Bathon et al., 2000; Feldmann and Maini, 2001). However, the concern of using anti-TNF treatment is that it can be associated with an increased risk of developing serious infections, due to the important role that TNF plays in regulating immune responses (Desai and Furst, 2006; Ellerin et al., 2003). TNF has been reported to be required for
normal innate and adaptive immune response (see instruction and Table 6.1) and play essential roles in host defense against many pathogens, such as *mycobacterium tuberculosis* (Flynn et al., 1995), HBV (Kasahara et al., 2003), and HIV (Herbein et al., 1996). So it is not surprising that disruption of TNF function may increase the susceptibility of the host to certain pathogens. However, according to the data collected from clinical trial and post-marketing analysis, the treatment with TNF blocking reagents is relatively well tolerated (Calabrese et al., 2004; Desai and Furst, 2006). There is only a slight increase in the risk of non-serious infections and no consistent evidence suggesting an increased risk in serious infections. As mentioned previously, TNF plays a role in decreasing HBV replication (Desai and Furst, 2006; Guidotti et al., 1996), so the blocking of TNF signal may lead to the increase of viral loads in patients with chronic HBV infection. Indeed, among eleven HBV patients who have been treated with anti-TNF therapies, only two patients show reactivation of HBV (Desai and Furst, 2006). Additionally, HIV patients receiving anti-TNF treatment also showed a very low frequency of increase in viral loads. One study reported that sixteen out of seventeen HIV patients receiving single dose of anti-TNF reagent showed no effect on either plasma HIV RNA level or CD4 T cell counts. Another report of 13 patients with HIV and active TB showed that a 4-week anti-TNF treatment had no effect on these patients, either. However, one out of three HIV patients with RA receiving anti-TNF treatment experienced repeated infection, although the CD4 T cell counts and HIV RNA level were stable during the treatment. The difference
### Table 6.1 Influences of TNF on immune cells

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<td><strong>Activate</strong></td>
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<td><strong>Autoinduce TNF-α</strong></td>
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<td><strong>Chemotaxis and migration</strong></td>
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<td><strong>Inhibit differentiation</strong></td>
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<th>Lymphocytes</th>
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<td><strong>Induce T cell colony formation</strong></td>
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<td><strong>Induce superoxide in B cells</strong></td>
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<td><strong>Induce apoptosis in mature T cells</strong></td>
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<td><strong>Activate cytotoxic T cell invasiveness</strong></td>
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This table is adapted from Ellerin 2003 Arthritis Rheum. *48*:3013-22
between this patient and the other two was that this patient had advanced HIV infection with CD4 T cell counts less than 50/mm³ (Calabrese et al., 2004). Therefore, an immune system with a normal T cell pool is very important for controlling infections during anti-TNF treatment.

Our data shown here indicate that although TNF is important for naïve mice to control VV infection, the heterologous protective immunity mediated by LCMV-immunity completely compensates the need for TNF on defending against VV infection. This result implies that the anti-TNF therapies in treating various human diseases may be relatively safe, perhaps in large part due to heterologous immunity. Humans are exposed to many pathogens throughout their lives and have a large complex pool of memory T cells that have the potential to cross-react with any new pathogen and mediate heterologous protective immunity.

F. Future direction

Our data shown above suggest that Fas/FasL signaling is responsible for the generation of panniculitis in LCMV-immune mice during VV infection. In the future we want to test whether panniculitis in VV-infected LCMV-immune mice is induced exclusively by FasL expressed on the cross-reactive LCMV-specific memory T cells. To test this possibility, we can adoptively transfer CFSE-labeled splenocytes from one LCMV-immune gld mouse into one WT and one gld naïve host. At the
same time, splenocytes derived from one WT LCMV-immune mouse will be transferred into one WT and one gld host. All hosts will then be challenged with VV. At day 6 of infection, we will determine the proliferation of LCMV-specific T cells by loss of CFSE label, and record the levels of panniculitis in these hosts. According to our data (Fig 5.4A), gld hosts that received gld LCMV-immune cells will have no increased levels of panniculitis, while WT hosts receiving WT donor cells will show enhanced panniculitis. By comparing the levels of panniculitis shown in gld hosts receiving WT donor cells and WT hosts receiving gld donor cells to these two control groups respectively, we should be able to tell whether the expression of FasL on activated VV-specific T cells is also involved in the pathogenesis of panniculitis.

To further understand why cross-reactive LCMV-specific memory T cells can induce altered immunopathology during VV infection, we would like to test two possibilities. First, compared to VV-specific memory T cells, cross-reactive LCMV-specific memory T cells may have lower affinity to VV antigens and result in a not very efficient protection (partial protection) but strong inflammation, which leads to the generation of panniculitis. To test whether LCMV-immune memory cells can induce higher levels of inflammation, we can first test the levels of inflammatory cytokines, such as IL-1, IL-6 and TNF, as well as immune regulatory cytokines, including IL-10, TGF-β and IL-17 in the fad pads and serum of VV-infected hosts that are transferred with either LCMV-immune cells or
VV-immune cells. We can also test whether there is a correlation between the levels of those cytokines and panniculitis. To test whether the pathogenesis of panniculitis is due to inefficient protection during early VV infection, we can transfer LCMV memory T cells from one single donor into two naïve hosts. According to the data shown in Chapter IV, we can suppose these two naïve hosts will have similar levels of panniculitis. One of the hosts will be used to test VV loads at day 3 of VV infection, while the other one will be sacrificed at day 6 of infection to record the levels of panniculitis. With a number of donor-recipient pairs, we should be able to find whether there is a correlation between the VV titer (day 3) and levels of panniculitis (day 6). To directly test whether the pathogenesis of panniculitis is caused by cross-reactive memory T cells with lower affinity, at day 6 of VV infection, the lymphocytes will be isolated from multiple organs of LCMV-immune mice. As we mentioned earlier, VV-a11r can induce cross-reactive responses from T cells that are specific to LCMV-GP34, NP205 or GP118. Isolated cells will be double stained with VV-a11r tetramer and LCMV-GP34, NP205 and GP118 tetramer, respectively. The MFI of VV-a11r will be evaluated in the double positive cross-reactive T cell populations. If panniculitis is mediated by lower affinity cross-reactive LCMV memory T cells, the mouse that generates higher levels of panniculitis will have lower MFI of VV-a11r in the cross-reactive CD8 T cell population.

Another possible explanation for the induction of panniculitis in LCMV-immune mice during VV infection is that there is a pathogenic
cross-reactive response selectively expanded. After analyzing more than one hundred VV-infected LCMV-immune mice, we found there was a very slight trend that mice using NP205 cross-reactive response tend to have lower levels of panniculitis (data not shown). To test whether there is a pathogenic epitope responsible for the pathogenesis of panniculitis, we can immunize mice with different LCMV variants and test the levels of pathology in these mice after VV infection. There is one LCMV variant (GPNPV) having partial deletions of their responses to all four dominant epitopes (NP396, GP33/34, GP207). There is also a LCMV-NP205 variant with a single mutation within the NP205 epitope, which leads to a decreased NP205-specific response in mice immunized with this variant. After immunizing mice with LCMV or its variants, these mice will be challenged with VV and the levels of panniculitis will be recorded at day 6 of infection. The levels of proliferation of T cells that are specific to all five LCMV epitopes will be examined. If there is a pathogenic epitope, there will be a correlation between the levels of panniculitis and the levels of the proliferation of T cells specific to that pathogenic epitope. To further confirm this observation, mice can be immunized with different LCMV peptides and then challenged with VV. The mice immunized with the pathogenic epitope will tend to have higher levels of panniculitis, while the mice immunized with the non-pathogenic epitope will show lower levels or no panniculitis.
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