The Virus-Specific CD4+ T Cell Response During Acute Lymphocytic Choriomeningitis Virus Infection and into Long Term Memory: a Dissertation

Steven Michael Varga
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THE VIRUS-SPECIFIC CD4 T CELL RESPONSE DURING ACUTE LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION AND INTO LONG TERM MEMORY

A Dissertation Presented

By

STEVEN MICHAEL VARGA

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

JANUARY 1999

IMMUNOLOGY AND VIROLOGY
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THE VIRUS-SPECIFIC CD4⁺ T CELL RESPONSE DURING ACUTE LYMPHOCYTIC CHORIOMENINGITIS VIRUS INFECTION AND INTO LONG TERM MEMORY

A Dissertation Presented By

STEVEN MICHAEL VARGA

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Program in Immunology and Virology
January 1999
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I would like to dedicate this work to my parents, especially my dad, who suffered a debilitating stroke shortly after I started my thesis research. I must also thank my wife Cathryn, who has made me smile from the day we met. She has given me nothing but love and support throughout my time here without asking for anything in return.

I would like to thank my mentor Dr. Ray Welsh for allowing me to pursue my doctoral studies in his laboratory. From the very beginning Ray has given me the freedom to pursue my ideas and has challenged me to become a better scientist.

I would also like to thank several people without whose contributions this work would not have been possible; all the members of the FACS facility, especially Tammy Krumpoch and Barbara Fournier, and the members of my dissertation committee, Drs. Ennis, Greiner, Woda, Ignotz, and Robinson.

Finally, I would like to thank the members of the Welsh laboratory for their help, constant support, and friendship, especially Dr. Liisa Selin, Carey O’Donnell and Keith Daniels. I have never had the privilege of working with a better group of people. Thanks.
CD4⁺ T cells play a central role in immunity. During virus infections, CD4⁺ T cells provide the necessary help for B cells to secrete anti-viral antibody and may act as effector cells themselves through the secretion of anti-viral cytokines such as IFN-γ and TNF-α. Recent studies in the lymphocytic choriomeningitis virus (LCMV) system have shown that CD4⁺ T cells are required to maintain the clearance of persistent viral infections as well as maintain virus-specific memory CD8⁺ cytotoxic T lymphocytes (CTL). Despite these important functions, surprisingly little information exists concerning the longevity, magnitude, and stability of the CD4⁺ T cell response following a virus infection. This thesis takes advantage of the well-studied LCMV system to address the above issues as well as to examine the role CD4⁺ T cells play during heterologous virus infections and to determine the fate of CD4⁺ T cells following a high-dose LCMV infection.

The cell surface phenotype of the CD4⁺ T cells was first examined in C57BL/6 mice acutely infected with LCMV. FACS analysis revealed the modulation of several activation markers on CD4⁺ T cells during an acute infection with LCMV, consistent with an activated cell phenotype. In addition, 25% of the CD4⁺ T cells were blast-sized by day 7 post-infection (p.i.) even though the total number of CD4⁺ T cells did not increase in the spleen during the acute infection. Additional studies were performed using CZ-1, a
novel monoclonal antibody (mAb) previously generated in our laboratory that defines a sialic acid-dependent CD45RB-associated epitope. Examination of the expression of the CZ-1 antigen on CD4+ T cells following LCMV infection revealed that the blast-sized CD4+ T cells at day 6 p.i. were CZ-1+. Further cell surface phenotyping showed that those blast cells activated at day 6 p.i. were CD45RBloCD44hiCD62L+. This contrasts with the CZ-1'CD45RBhiCD44loCD62L+ resting cell population prior to infection. To determine if memory CD4+ T cells continued to express the CZ-1 epitope long after resolution of the LCMV infection, CD4+CZ-1+ and CD4+CZ-1- populations were purified by cell sorting and placed into an in vitro proliferation assay with LCMV-infected antigen-presenting cells (APC). It was found that the CD4+CZ-1+ population contained virtually all of the virus-specific memory. Thus, these studies indicate that the CZ-1 epitope defines a novel activation and memory marker for murine CD4+ T cells.

Examination of virus-specific cytokine production using ELISPOT assays showed a significant increase in the number of IFN-γ-secreting cells in the spleen during an acute LCMV-infection. CD8+ T cells made up the majority of the IFN-γ-producing cells, but analysis of the cell culture supernatants by ELISA revealed that the CD4+ T cells produced more IFN-γ on a per cell basis. No significant increase in IL-4 levels was detected under these experimental conditions. These data suggest that LCMV infection induces primarily a virus-specific Th1 response that is characterized by increased IFN-γ production.
No quantitative information was known about the frequency and longevity of the LCMV-specific CD4+ T cell response. Using limiting dilution assays (LDA), I examined the CD4+ T cell precursor (Thp) frequency in C57BL/6 mice infected with LCMV. The virus-specific CD4+ Thp frequency increased from <1/100,000 in uninfected mice to a peak of approximately 1/600 in FACS-purified splenic CD4+ T cell populations by 10 days p.i. with LCMV. After the peak of the response, the CD4+ Thp frequency decreased only about 2-fold per CD4+ T cell to approximately 1/1200 and remained stable into long-term memory. The CD4+ Thp frequency to each of the two known LCMV major histocompatibility complex (MHC) class II-restricted peptides dropped only 2- to 7-fold from the peak of the acute LCMV response into long-term memory. Thus, the CD4+ T cell frequencies remain elevated after the acute infection subsides and remain extremely stable throughout long-term immunity.

The above results show that LDA can account for <1% of the CD4+ T cells as being virus-specific following LCMV infection. However, using newer, more sensitive assays based on intracellular cytokine production, >20% of the CD4+ T cells secreted IFN-γ after stimulation with phorbol myristic acid and ionomycin during the peak of the acute CD4+ T cell response. In addition, >10% of the CD4+ T cells secreted IFN-γ after stimulation with the LCMV MHC class II-restricted CD4 peptides. Thus, these new sensitive assays reveal a heretofore unappreciated, yet profound antigen-specific CD4+ T cell response during LCMV infection.
Infection of mice with a series of unrelated viruses, termed heterologous viruses, causes the reduction of memory CD8⁺ T cells specific to earlier infections. In order to examine the fate of CD4⁺ T cells under these conditions, I examined cytokine production and followed the CD4⁺ T cell frequency following heterologous virus infections. Challenge of LCMV-immune mice with vaccinia virus (VV) resulted in a significant increase in both the amount of IFN-γ protein and the frequency of IFN-γ-producing cells in the peritoneal cavity 3 days after infection as compared to control non-immune mice acutely infected with VV or to LCMV-immune mice alone. Intracellular IFN-γ staining revealed that both CD4⁺ and CD8⁺ T cells contributed to this increased IFN-γ production. LDA analysis of the LCMV-specific CD4⁺ T cell frequency following multiple heterologous virus infections or protein antigen immunizations, revealed that the CD4⁺ T cell frequency remains stable even under conditions that reduce the LCMV-specific CD8⁺ CTLp frequency. Additional studies using high-dose LCMV Clone 13 demonstrated that, like CD8⁺ T cells, there is a decline in detectable LCMV-specific CD4⁺ T cell during overwhelming virus infections.

The data presented in this thesis help provide a better understanding of the CD4⁺ T cell response during virus infections. I make several novel observations, including the demonstration that mAb CZ-1 defines a novel activation and memory marker for CD4⁺ T cells, that the LCMV-specific memory CD4⁺ T cell frequency remains extremely stable into long-term immunity, and that heterologous virus infections do not disturb the stable
memory CD4+ T cell pool following a virus infection. I also provide data using new sensitive assays based on intracellular cytokine production that there is a much more profound antigen-specific CD4+ T cell response during viral infections than has previously been realized. Finally, I provide evidence that the virus-specific CD4+ T cells become unresponsive following a high-dose LCMV Clone 13 infection. Thus, the data presented in this thesis highlight some important similarities and differences between the CD4+ and CD8+ T cell responses during acute viral infections.
TABLE OF CONTENTS

COPYRIGHT NOTICE............................................................................................................ ii

ACKNOWLEDGMENTS......................................................................................................... iv

ABSTRACT.......................................................................................................................... v

TABLE OF CONTENTS........................................................................................................... x

LIST OF TABLES.................................................................................................................. xvi

LIST OF FIGURES.............................................................................................................. xviii

ABBREVIATIONS..................................................................................................................xxi

CHAPTER I: INTRODUCTION.............................................................................................. 1

A. ANTIGEN RECOGNITION AND ACTIVATION OF T CELLS............................................. 1

B. CD4+ T CELL SUBSETS................................................................................................. 5

C. ROLE OF CD4+ T CELL SUBSETS IN VIVO................................................................. 7

D. CD4+ T CELL MEMORY.............................................................................................. 10

E. CD4+ T CELL RESPONSE TO VIRUSES...................................................................... 15

F. ACUTE T CELL RESPONSE TO LCMV...................................................................... 20

G. T CELL MEMORY FOLLOWING LCMV INFECTION..................................................... 23

H. THESIS OBJECTIVES.................................................................................................. 24
CHAPTER II: MATERIALS AND METHODS........................................................................................27
A. MICE..............................................................................................................................................27
B. VIRUSES, PROTEIN ANTIGENS, AND INOCULATIONS OF MICE...........................................27
C. PREPARATION OF CELLS..............................................................................................................28
D. ANTIBODIES AND REAGENTS......................................................................................................29
E. PLAQUE ASSAYS..........................................................................................................................30
F. CENTRIFUGAL ELUTRIATION........................................................................................................30
G. FLOW CYTOMETRY AND CELL SORTING....................................................................................31
H. LIMITING DILUTION ASSAY OF VIRUS-SPECIFIC PRECURSORS..............................................33
I. PROLIFERATION ASSAYS..............................................................................................................35
J. INTRACELLULAR CYTOKINE STAINING......................................................................................36
K. CYTOKINE ELISA.........................................................................................................................37
L. CYTOKINE ELISPOT ASSAYS........................................................................................................39
M. STATISTICAL ANALYSIS................................................................................................................40

CHAPTER III: VISUALIZATION OF THE CD4+ T CELL RESPONSE FOLLOWING LCMV INFECTION..................................................................................................................41
A. T CELL DISTRIBUTION AND ACTIVATION PHENOTYPE OF CD4+ T CELLS DURING THE ACUTE LCMV INFECTION AND ON INTO MEMORY..................................................42
B. CHARACTERIZATION OF CZ-1 AS A NOVEL CD4+ T CELL ACTIVATION MARKER..........................................................................................................................51
C. CZ-1 ALSO SERVES AS A MEMORY MARKER FOR VIRUS-INDUCED CD4+ T CELLS..........................................................................................................................55
D. CYTOKINE SECRETION DURING THE ACUTE LCMV INFECTION..............65
E. CD4+ T CELLS PRODUCE MORE IFN-γ THAN CD8+ T CELLS....................70
F. BRIEF SUMMARY OF CHAPTER III............................................................................74

CHAPTER IV: EXAMINATION OF THE LCMV-SPECIFIC CD4+ T CELL FREQUENCY FROM ACUTE INFECTION INTO MEMORY.........................................................76
A. QUANTITATION OF THE CD4+ THP FREQUENCY DURING THE ACUTE LCMV INFECTION INTO MEMORY..................................................................................77
B. QUANTITATION OF THE PEPTIDE-SPECIFIC CD4+ THP FREQUENCY FROM THE PEAK OF THE ACUTE INFECTION INTO LONG-TERM MEMORY..........................................................................................................................83
C. PHENOTYPE OF LCMV-SPECIFIC CD4+ THP.....................................................85
D. INTRACELLULAR STAINING OF CYTOKINE-PRODUCING CD4+ T CELLS..........................................................................................................................90
E. INTRACELLULAR STAINING OF PEPTIDE-SPECIFIC IFN-γ-PRODUCING CD4+ T CELLS..........................................................................................................................92
F. BRIEF SUMMARY TO CHAPTER IV .................................................................102

CHAPTER V: STABILITY OF THE LCMV-SPECIFIC CD4+ T CELL
FREQUENCY FOLLOWING HETEROLOGOUS VIRUS INFECTIONS ..........103

A. T CELL DISTRIBUTION AND ACTIVATION PHENOTYPE OF CD4+ T CELLS
FOLLOWING DIFFERENT VIRUS INFECTIONS (LCMV, PV, VV, MCMV) ..............................................................104

B. INCREASED FREQUENCY OF IFN-γ PROTEIN AND IFN-γ-SECRETING CELLS
FOLLOWING A HETEROLOGOUS VIRUS INFECTION ......................106

C. LCMV-SPECIFIC CD4+ THP FREQUENCY DOES NOT DECLINE FOLLOWING
MULTIPLE HETEROLOGOUS VIRUS INFECTIONS .........................110

D. LCMV-SPECIFIC CD4+ THP FREQUENCY FOLLOWING PROTEIN ANTIGEN
IMMUNIZATION ..................................................................................117

E. INTRACELLULAR STAINING OF PEPTIDE-SPECIFIC IFN-γ-PRODUCING
CD4+ T CELLS FOLLOWING MULTIPLE HETEROLOGOUS VIRUS
INFECTIONS ........................................................................................119

F. INTRACELLULAR STAINING OF CYTOKINE-PRODUCING CD4+ T CELLS
FOLLOWING ACUTE LCMV, PV, VV, OR MCMV INFECTION ............125

G. BRIEF SUMMARY TO CHAPTER V ......................................................125
CHAPTER VI: EXHAUSTION OF LCMV-SPECIFIC CD4+ T CELLS FOLLOWING HIGH DOSE LCMV INFECTION
A. QUANTITATION OF THE CD4+ THP FREQUENCY FOLLOWING LCMV CLONE 13 INFECTION
B. INTRACELLULAR STAINING OF PEPTIDE-SPECIFIC IFN-γ-PRODUCING CD4+ T CELLS FOLLOWING LCMV CLONE 13 INFECTION
C. BRIEF SUMMARY TO CHAPTER VI

CHAPTER VII: DISCUSSION
A. ACTIVATION PHENOTYPE OF CD4+ T CELLS AND CHARACTERIZATION OF MAB CZ-1
B. ANALYSIS OF THE CYTOKINE RESPONSE DURING LCMV INFECTION
C. DETECTION OF A HIGH FREQUENCY OF LCMV-SPECIFIC CD4+ T CELLS
D. STABILITY OF LCMV-SPECIFIC CD4+ THP INTO LONG-TERM IMMUNITY
E. ROLE OF CD4+ T CELLS DURING HETEROLOGOUS VIRUS INFECTIONS
F. EXHAUSTION OF VIRUS-SPECIFIC CD4+ T CELLS
G. CONCLUSIONS
CHAPTER VIII: REFERENCES

160
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-1</td>
<td>Mean fluorescence intensity of activation markers on CD4(^+) T cells during the acute LCMV infection and on into memory</td>
</tr>
<tr>
<td>III-2</td>
<td>Frequency of IFN-(\gamma)- and IL-4-secreting cells on day 7 post-infection with LCMV</td>
</tr>
<tr>
<td>III-3</td>
<td>Frequency of IFN-(\gamma)-secreting cells and the quantity of IFN-(\gamma) secreted into the supernatant by sorted CD4(^+) and CD8(^+) T cells during the acute LCMV infection</td>
</tr>
<tr>
<td>IV-1</td>
<td>LCMV-specific Thp frequencies during the acute infection and on into memory</td>
</tr>
<tr>
<td>IV-2</td>
<td>Effect of anti-CD4 or anti-IL-4 mAb on LCMV-specific Thp frequencies</td>
</tr>
<tr>
<td>IV-3</td>
<td>LCMV-specific Thp frequencies to the LCMV MHC class II-restricted peptides</td>
</tr>
<tr>
<td>IV-4</td>
<td>Phenotype of LCMV-immune CD4(^+) T cell precursors</td>
</tr>
<tr>
<td>IV-5</td>
<td>Frequency of cytokine-secreting CD4(^+) T cells during the acute LCMV infection following stimulation with the LCMV MHC class II-restricted peptides in the presence of IL-2 and monensin</td>
</tr>
<tr>
<td>V-1</td>
<td>LCMV-immune mice challenged with heterologous viruses..........................114</td>
</tr>
<tr>
<td>V-2</td>
<td>PV-immune mice challenged with heterologous viruses..........................115</td>
</tr>
<tr>
<td>V-3</td>
<td>VV-immune mice challenged with heterologous viruses..........................116</td>
</tr>
<tr>
<td>V-4</td>
<td>LCMV-immune mice challenged with KLH in CFA................................120</td>
</tr>
<tr>
<td>V-5</td>
<td>LCMV-immune mice challenged with KLH in CFA and OVA in Titer Max adjuvant....................................................................................................................122</td>
</tr>
<tr>
<td>V-6</td>
<td>Frequency of IFN-γ-producing peptide-specific CD4⁺ T cells following heterologous virus infections..................................................................................................................124</td>
</tr>
<tr>
<td>VI-1</td>
<td>CD4⁺ T cell precursor frequencies following LCMV Clone 13 infection.................................................................................................................................129</td>
</tr>
<tr>
<td>VI-2</td>
<td>Expression of activation markers on CD4⁺ T cells during the acute LCMV Clone 13 infection..............................................................................................................133</td>
</tr>
<tr>
<td>VI-3</td>
<td>Increase in the percentage of apoptotic CD4⁺ T cells during the acute LCMV infection with either Armstrong or Clone 13.................................................135</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-1</td>
<td>43</td>
<td>Phenotype of splenic cell populations during an acute LCMV infection of C57BL/6 mice.</td>
</tr>
<tr>
<td>III-2</td>
<td>45</td>
<td>Expression of CD62L and CD44 on CD4(^+) T cells during LCMV infection.</td>
</tr>
<tr>
<td>III-3</td>
<td>48</td>
<td>Expression of CD25 and CD69 on CD4(^+) T cells during LCMV infection.</td>
</tr>
<tr>
<td>III-4</td>
<td>49</td>
<td>Expression of CD44, CD62L, and CD69 on CD4(^+) T cells during LCMV infection.</td>
</tr>
<tr>
<td>III-5</td>
<td>53</td>
<td>Expression of CZ-1 and CD45RB on splenocytes during LCMV infection.</td>
</tr>
<tr>
<td>III-6</td>
<td>54</td>
<td>Coexpression of CZ-1 and CD45RB on splenocytes during an acute LCMV infection.</td>
</tr>
<tr>
<td>III-7</td>
<td>56</td>
<td>Surface phenotype of blast-sized cells during an LCMV infection.</td>
</tr>
<tr>
<td>III-8</td>
<td>57</td>
<td>Expression of CD44 and CD62L on blast-sized CD4(^+) T cells during an acute LCMV infection.</td>
</tr>
<tr>
<td>III-9</td>
<td>58</td>
<td>Comparison of CD44 and CD62L expression with the CZ-1 antigen.</td>
</tr>
<tr>
<td>III-10</td>
<td>59</td>
<td>Expression of CZ-1 on splenocytes from an LCMV-immune animal.</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>III-11</td>
<td>LCMV-specific memory CD4+ T cells express the CZ-1 antigen............61</td>
<td></td>
</tr>
<tr>
<td>III-12</td>
<td>Predominantly CD4+ T cells grow out in the proliferation cultures...........62</td>
<td></td>
</tr>
<tr>
<td>III-13</td>
<td>Small CD4+ T cells in LCMV-immune mice express the CZ-1 antigen........64</td>
<td></td>
</tr>
<tr>
<td>III-14</td>
<td>Frequency of cytokine-producing cells in LCMV-infected C57BL/6 mice.................................................................69</td>
<td></td>
</tr>
<tr>
<td>IV-1</td>
<td>Regression lines for purified CD4+ T cells recovered from the spleens of C57BL/6 mice 7 and 60 days p.i. with LCMV.......78</td>
<td></td>
</tr>
<tr>
<td>IV-2</td>
<td>Intracellular IFN-γ and IL-4 expression of virus-induced CD4+ T cells following stimulation with PMA and ionomycin........91</td>
<td></td>
</tr>
<tr>
<td>IV-3</td>
<td>Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells..94</td>
<td></td>
</tr>
<tr>
<td>IV-4</td>
<td>CD44 expression on peptide-specific CD4+ T cells staining positive for intracellular IFN-γ.................................................................95</td>
<td></td>
</tr>
<tr>
<td>IV-5</td>
<td>Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells during an acute LCMV infection into memory........................96</td>
<td></td>
</tr>
<tr>
<td>IV-6</td>
<td>Intracellular IFN-γ and IL-4 expression of LCMV peptide-specific CD4+ T cells from CD8 knock-out mice........................97</td>
<td></td>
</tr>
<tr>
<td>V-1</td>
<td>CD4/CD8 ratio during acute LCMV, VV, PV, or MCMV infection........105</td>
<td></td>
</tr>
<tr>
<td>V-2</td>
<td>Increased IFN-γ protein levels in peritoneal lavage fluid of LCMV-immune mice versus control-immune mice 3 days after challenge with VV......108</td>
<td></td>
</tr>
</tbody>
</table>
V-3 Increased numbers of IFN-γ-secreting CD8+ and CD4+ T cells in the peritoneum of LCMV-immune mice challenged with VV (day 3) 109

V-4 Reduction of CD8+ CTLp but not CD4+ Thp following challenge of LCMV-immune mice with several heterologous virus infections 111

V-5 Reduction of CD8+ CTLp but not CD4+ Thp following challenge of PV+LCMV-immune mice with several heterologous virus infections 113

V-6 Intracellular IFN-γ expression of virus-induced CD4+ T cells following stimulation with PMA and ionomycin 126

VI-1 Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells following LCMV Clone 13 or Armstrong infection 131

VI-2 Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells from LCMV Clone 13 infected C57BL/6 mice 132
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney (cells)</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund's adjuvant</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLp</td>
<td>cytotoxic T lymphocyte precursor</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISpot</td>
<td>enzyme-linked immunospot assay</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LDA</td>
<td>Limiting dilution analysis</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>MHV-68</td>
<td>Murine gammaherpesvirus-68</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneal exudate cell</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PV</td>
<td>Pichinde virus</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post-infection</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>Thp</td>
<td>T helper cell precursor</td>
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<tr>
<td>TMG</td>
<td>TiterMax Gold (adjuvant)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>[3H]TdR</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNF-β</td>
<td>tumor necrosis factor beta</td>
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<tr>
<td>VV</td>
<td>vaccinia virus</td>
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<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
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A. Antigen recognition and activation of T cells. The immune system has evolved several mechanisms to defend the host against foreign antigens. The immune response can be broadly divided into two arms, the humoral immune response that is characterized by antibodies produced by B cells, and the cell-mediated immune response that is characterized by the various actions of T cells. Both of these arms of the immune system play vital roles in protecting the host from pathogens, including viruses (Doherty and Kaufmann, 1994; Sher and Coffman, 1992). Anti-viral antibodies can bind to and inactivate extracellular virions as well as mediate the destruction of infected cells through antibody-dependent mechanisms. Both of these mechanisms allow antibodies to limit the spread of the virus in the host. However, during many virus infections the actions of T cells are necessary to destroy the majority of already infected cells. Thus, T cells play a central role in the immune response to many virus infections (Doherty et al., 1992; Zinkernagel, 1996).

The majority of mature T cells express a T cell receptor (TCR), which is a membrane-bound heterodimer composed of α and β chains (Fields and Mariuzza, 1996). T cells that express α/β TCR can be divided into cells expressing either CD4 or CD8 surface antigens.
T cells bearing the CD4 marker are generally considered to comprise the helper component of the immune system, whereas T cells bearing the CD8 marker are cytotoxic cells capable of destroying infected cells in the body.

The T cell response to a virus infection begins with the activation and expansion of virus-specific T cell populations. Antigen-specific T cell activation is dependent on a series of complex cell-cell interactions between T cells and antigen presenting cells (APC), which express major histocompatibility complex (MHC) proteins (Germain, 1994). The MHC proteins are highly polymorphic cell surface glycoproteins that bind antigenic peptides within the cell and present them at the cell surface. CD8$^+$ T cells recognize peptides bound to MHC class I molecules, which are expressed by all nucleated cells in the body (Schwartz, 1985). Most MHC class I proteins bind, in the endoplasmic reticulum, to peptides that are derived from endogenously synthesized proteins. Crystal structures of MHC class I molecules complexed with peptides have demonstrated that the MHC class I peptide-binding groove is closed at both ends and that the NH$_2$- and COOH-terminal ends of the peptide form hydrogen bonds with residues within the pocket of the binding groove (Germain, 1994). MHC class I molecules require the peptide for proper folding and due to these interactions, the length of peptides bound to MHC class I molecules is limited to 8-10 amino acids. In contrast, CD4$^+$ T cells recognize peptides bound to MHC class II molecules. MHC class II proteins are constitutively expressed on a limited number of bone marrow-derived cell populations,
such as macrophages, dendritic cells, and B cells. Since these are the only cells in the body that express constitutive levels of both MHC class I and class II proteins, these cell populations are often referred to as professional APC (Brown et al., 1993). Newly synthesized MHC class II molecules are complexed with an invariant chain that prevents peptide-binding to the MHC and targets the class II molecules to late endocytic compartments where, after disassociation with the invariant chain, these molecules can bind antigenic peptides (Germain, 1994; Schwartz, 1985). Most of these peptides are derived from exogenous proteins that are internalized by the APC. In contrast to MHC class I molecules, MHC class II proteins are not dependent on peptides for proper folding, and their peptide-binding groove is open at both ends (Sadegh-Nasseri and Germain, 1992; Stern et al., 1994). These characteristics allow MHC class II molecules to present peptides from 10-35 amino acids in length. Thus, T cell activation begins with the recognition of antigenic peptides bound to MHC molecules on APC by T cells bearing the appropriate antigen-specific TCR.

Activation of naive T cells requires two signals. Signal one is received through the TCR after engaging an antigen-MHC complex on an APC (Schwartz, 1985). The second "costimulatory" signal is provided by one or more distinct APC surface molecules interacting with coreceptors on the T cells (Mondino and Jenkins, 1994). The TCR and other cell surface molecules initiate T cell activation by transducing signals across the plasma membrane of the T cell as well as increasing the avidity of the T cell-APC
interaction. These signals induce the transcription of several genes in the T cell, including those encoding interleukin 2 (IL-2) and the IL-2 receptor, and also induce a progression from G₀ to the G₁ phase of the cell cycle. The subsequent production of IL-2, its secretion, and binding to the IL-2 receptor, are thought to signal the cell to progress from G₁ into S (Mills et al., 1993; Nakamura et al., 1993). Naive T cells that receive signals through the TCR in the absence of proper costimulatory signals are induced into a state of unresponsiveness, termed anergy (Matzinger, 1994). This failure of T cells to proliferate in response to cross-linking of the TCR alone stems from their inability to secrete IL-2. Thus, following T cell activation, IL-2 acts as a strong growth factor promoting the expansion of the activated T cell population. Following additional exposures to antigen, IL-2 secreting T cells will differentiate into cells capable of secreting multiple cytokines, termed T helper 0 (Th0) cells (Elson et al., 1995; Firestein et al., 1989).

During a virus infection, these virus-specific T cells will undergo further expansion and continued differentiation into effectors until the virus is eliminated. Once the virus is cleared, the expanded effector T cell population goes through a contraction phase, leaving behind a stable pool of memory T cells that provide long-term immunity (Ahmed and Gray, 1996; Doherty et al., 1996; Welsh et al., 1995). The mechanisms that determine and regulate this transition from effector to memory T cells are poorly understood.
B. CD4\(^+\) T cell subsets. As mentioned above, following initial stimulation, naive T cells secrete primarily IL-2. With further stimulation, these cells differentiate into effector cells capable of secreting a wide range of cytokines (Bradley et al., 1991). Upon closer examination of the cytokines secreted by CD4\(^+\) T cell clones, it was noticed that the clones would often secrete distinct patterns of cytokines. This important observation led to the separation of CD4\(^+\) T cells into Th1 and Th2 subsets (Mosmann et al., 1986; Mosmann and Coffman, 1989; Mosmann et al., 1991). Th1 cells produce IL-2, interferon gamma (IFN-\(\gamma\)), and lymphotoxin (tumor necrosis factor beta, TNF-\(\beta\)), whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Mosmann et al., 1986; Mosmann and Coffman, 1989; Mosmann and Moore, 1991; Openshaw et al., 1995). Several other cytokines are not tightly restricted to either subset and are often secreted by both Th1 and Th2 cells. These include IL-3, tumor necrosis factor alpha (TNF-\(\alpha\)), and granulocyte-macrophage colony-stimulating factor (Mosmann and Coffman, 1989). T cells producing cytokines typical of both Th1 and Th2 clones have also been described in murine and human systems. These have been termed Th0 cells, and may represent the precursors of the polarized Th1 and Th2 phenotypes (Elson et al., 1995; Firestein et al., 1989; Miner and Croft, 1998; Sad and Mosmann, 1994). A fourth subset of CD4\(^+\) T cells, termed Th3, has recently been proposed for CD4\(^+\) T cells that secrete high levels of transforming growth factor beta (TGF-\(\beta\)). These cells may play important roles in downregulating immune responses and may represent the suppressor T cell population,
the presence of which has long been suggested in the literature (Letterio and Roberts, 1998; Sanders et al., 1988). Although the Th1 and Th2 subsets were initially reported using mouse T cell clones, this distinction has now been shown using CD4+ and CD8+ T cells from infected or uninfected mice and in cells derived from humans (Biron, 1994; Firestein et al., 1989; Kagi et al., 1995; Launois et al., 1995; Romagnani, 1991; Sad and Mosmann, 1994; Sher and Coffman, 1992).

*In vitro* studies examining CD4+ T cell differentiation have elucidated several of the factors required to develop polarized Th1 or Th2 cell populations. These studies have shown that the cytokine milieu present during the priming of naive CD4+ T cells determines the ultimate differentiation pathway that is followed. Thus, IL-12, IFN-γ, and TGF-β induce the differentiation of Th1 cells whereas IL-4 induces the development of Th2 cells (Bradley et al., 1996; Hsieh et al., 1992; Hsieh et al., 1993; Murphy, 1998; O'Garra, 1998; O'Garra and Murphy, 1994; Seder et al., 1993; Seder et al., 1992). Additional studies suggest that these polarized effector Th1 or Th2 cells may arise from a common naive T cell precursor that is uncommitted to either subset, i.e. the Th0 cells mentioned earlier (Miner and Croft, 1998; Nakamura et al., 1997; Sad and Mosmann, 1994). The cytokines secreted by these polarized Th1 and Th2 cells are also mutually inhibitory for the differentiation and effector functions of the reciprocal subset. IFN-γ selectively inhibits the cytokine production and proliferation of Th2 cells (O'Garra and Murphy, 1994), whereas IL-10 inhibits Th1 cytokine production and cell proliferation
(Fiorentino et al., 1991; Mosmann and Moore, 1991). In addition, recent work has shown that IL-4 can downregulate the expression of IL-12Rβ2 on Th2 cells, thus making them insensitive to IL-12 signals that would normally induce a Th1 cell response (Rogge et al., 1997; Szabo et al., 1997; Szabo et al., 1995). In addition to the potent effects of cytokines, other factors such as genetic background, costimulation, and antigen dose may also regulate the differentiation of Th1 and Th2 cells (Constant and Bottomly, 1997; Corry et al., 1994; Guler et al., 1997; Pfeiffer et al., 1991; Schountz et al., 1996). Recent studies have suggested that higher levels of antigen and costimulation result in Th2 differentiation (Constant et al., 1995; Hosken et al., 1995). There are currently no well-defined cell surface markers that can separate Th1 from Th2 cells. Even though most of these studies were initially performed using CD4+ T cells, many of these same observations have now been made using CD8+ T cells as well (Carter and Dutton, 1996; Erard et al., 1993; Noble et al., 1995; Sad et al., 1995).

C. Role of CD4+ T cell subsets in vivo. The functionally different subsets of CD4+ and CD8+ T cells that develop upon exposure to foreign antigen have important consequences for successful host defense. Many of the effector functions of T cells are mediated by the cytokines they secrete (Biron, 1994; O'Garra and Murphy, 1994). Through their production of IFN-γ and TNF-β, Th1 cells promote cell-mediated immune responses. Th1-derived cytokines promote cytotoxic and inflammatory responses,
activate macrophages to become more bactericidal, and induce delayed-type hypersensitivity (DTH) reactions (Mosmann et al., 1986; O'Garra and Murphy, 1994). In addition, Th1 cells can provide some B-cell help to produce antibodies, as IFN-γ is the major switch factor for antibodies of the IgG2a isotype in the mouse (IgG1 in the human). Antibodies of the IgG2a isotype promote phagocytosis by activating the classical complement pathway and binding to Fc receptors on macrophages. Thus, Th1 responses are often responsible for the eradication of intracellular pathogens (Biron, 1994; Sher and Coffman, 1992). In contrast, Th2 cells, through their secretion of IL-4, IL-5, and IL-10, provide help for B-cells to produce antibodies including the IgA, IgE, and IgG1 isotypes (IgG4 in humans) (Mosmann and Sad, 1996). Th2 cytokines also activate and recruit mast cells as well as induce eosinophil proliferation and differentiation (Sher and Coffman, 1992). Eosinophils are thought to play a direct role in causing inflammation of the airways and asthma. Thus, Th2 responses are generally associated with allergic or anti-inflammatory responses and are characterized by strong antibody responses. Therefore, Th1 cells favor cellular immunity whereas Th2 cells favor humoral immunity.

The regulation of the type of T cell response and other effector mechanisms chosen during an immune response can determine host susceptibility or resistance to many pathogens. The importance of Th1 versus Th2 responses in vivo initially became apparent in the experimental model of Leishmania major infection. In this system, resistant strains of mice, such as C57BL/6, mount a vigorous Th1-dominated response
whereas susceptible strains, such as Balb/c, generate a Th2-dominated response (Guler et al., 1996; Sher and Coffman, 1992). Furthermore, it was shown that susceptible mice could be made resistant by converting the Th2 response to a Th1 response by treating susceptible mice with anti-IL-4 monoclonal antibodies (mAb) (Bogen et al., 1993; Louis et al., 1998). In addition to Leishmania major, Th1 and Th2 cells play an important role in the immune response to several other pathogens. For example, Th1 responses are associated with protective immune responses in several mouse models of infection to intracellular bacteria, protozoa, and fungi. These include pathogens such as Trypanosama cruzi, Candida, Bordetella pertussis, and Listeria monocytogenes (Harty et al., 1996; Mencacci et al., 1998; Sher and Coffman, 1992). Th1 responses have also recently been associated with more severe disease in several mouse experimental models of autoimmune disease, whereas Th2 responses favor protection (Chen et al., 1994; Falcone and Bloom, 1997; Khoury et al., 1992; Peterson et al., 1992). Thus, Th2 responses are not always detrimental. In fact, protective Th2 responses are also seen in several mouse models of helminth infections including Trichuris muris, Nippostrongylus brasiliensis, and Schistosoma mansoni (Chensue et al., 1996; Metwali et al., 1996). In humans, subclinical Mycobacterium leprae infections are associated with strong DTH responses to mycobacterial antigens whereas disseminated leprosy is associated with a lack of DTH reaction and increased production of antibodies (Yamamura et al., 1997). In addition, recent studies have shown that humans with IL-12 receptor deficiencies are more
susceptible to both mycobacterial and *Salmonella* infections (Altare et al., 1998; de Jong et al., 1998). Thus, the cytokines produced by T cells and other cell types can play an important role in determining the outcome of many infectious diseases. However, there is only a limited amount of data concerning the effects of Th1 and Th2 subsets and their cytokines on immunity to virus infections.

**D. CD4+ T cell memory.** One important feature of the immune system is its ability to “remember” previously encountered antigens and generate a more rapid immune response which plays an essential role in protecting the host following a secondary exposure to the antigen. In functional terms, T cell memory can be characterized by an increased frequency of antigen-specific T cells that give a much larger, broader, and more accelerated response to antigen as compared to that elicited during a primary immune response (Swain, 1994). Thus, memory involves at least two types of change in T cells, clonal expansion through cell proliferation, and the differentiation of this expanded population into long-lived memory T cells (Dutton et al., 1998). One of the reasons that a secondary immune response is stronger than a primary immune response is that memory T cells specific for previously encountered pathogens can exist at frequencies 100-fold or greater than naive T cells. Memory T cells also differ qualitatively from naive T cells, and this may explain why the secondary immune response occurs more rapidly. In comparison to naive T cells, memory T cells have less stringent activation requirements.
which make them capable of responding to lower doses of antigen, a wider range of APC, as well as being activated by TCR signals in the absence of costimulation (Croft, 1994; Croft et al., 1994; Croft et al., 1992). These attributes may, at least in part, be due to the higher expression of several adhesion and activation molecules on memory T cells as compared to naive T cells, as discussed in more detail below.

Naive and memory T cells express a different pattern of cell surface markers that may be used to distinguish between the two populations. One such marker is CD45, a transmembrane tyrosine phosphatase found on the surface of all nucleated cells of hematopoietic origin (Thomas, 1989). Alternative splicing of three consecutive exons, named A, B, and C, generate eight different cell surface isoforms of CD45 that vary in molecular mass from 180-220 kDa (Johnson et al., 1989; Trowbridge, 1991). In addition, the extracellular domain of CD45 may be heavily glycosylated, further increasing the heterogeneity of these proteins (Trowbridge and Thomas, 1994). CD45 is expressed on all mature T cells, but different isoforms are expressed at various stages of T cell development and differentiation (Thomas and Lefrancois, 1988). CD45 isoform expression is used to identify CD4+ T cells at distinct stages of differentiation. For example, human CD4+ T cells can be divided into two populations based on staining with mAb to CD45RO and RA (Akbar et al., 1988; Smith et al., 1986). T cells recognized by anti-CD45RA mAb express the high molecular weight isoforms of CD45 and exhibit characteristics of naive T cells. In contrast, mAb directed against CD45RO stains T cells
expressing the lowest molecular weight isoform of CD45, and these cells are capable of responding to recall antigen (Bell, 1992; Beverley, 1992; Plebanski et al., 1992). Furthermore, CD45RA cells have been shown to convert to CD45RO cells upon activation in vitro (Byrne et al., 1988). Thus, in the human, naive T cells are CD45RA+ and CD45RO− whereas memory T cells are CD45RO+ and CD45RA+. However, studies have demonstrated a conversion of CD45RO+ cells to CD45RA+ cells in vivo. In addition, other work has shown that memory T cells capable of responding to recall antigens may be recovered from both CD45RA and CD45RO populations. These results suggest that memory T cells may regain the expression of markers associated with a naive cell phenotype.

In mice, the CD45 staining pattern for naive and memory T cells is not as clear cut due to the lack of an antibody specific for the murine CD45RO form. Some investigators have used the density of cell surface expression of CD45RB to distinguish naive and memory CD4+ T cells (Gray, 1993). Naive CD4+ T cells stain CD45RBhi, whereas memory CD4+ T cells stain CD45RBlo (Lee et al., 1990). However, the density of cell surface expression of CD45RB on T cells can change with activation or with cytokine treatment (Bunce and Bell, 1997).

Other differences in the expression of cell surface markers help distinguish naive and memory CD4+ T cell populations. Most of these other markers are adhesion molecules that play important roles in the trafficking of T cells throughout the body. The
proteoglycan CD44 (also known as Pgp-1) serves as a cell adhesion receptor, and its ligand, hyaluronate, is commonly expressed on extracellular matrices (Butcher and Picker, 1996). Memory CD4+ T cells express higher levels of CD44 than naive T cells (Butterfield et al., 1989). However, CD44 has limitations as a memory marker because its level of expression varies in different strains of mice (Budd et al., 1987; Lynch and Ceredig, 1989). The lymph node homing receptor, CD62L (also known as L-selectin and MEL-14 in mice) is another marker used to distinguish naive and memory T cells. CD62L regulates T cell migration by mediating lymphocyte attachment to the high endothelial venules of peripheral lymph nodes (Gallatin et al., 1983). Naive T cells are CD62L<sup>hi</sup> and lose expression following activation (Bradley et al., 1992; Bradley et al., 1991). However, CD62L<sup>lo</sup> phenotype may not be permanently stable, as some memory T cells appear to regain CD62L expression (Razvi et al., 1995). CD49d and CD11a (also known as LFA-1) are two other, though less well studied, molecules that are upregulated on memory T cells (Ewing et al., 1995). Each of these intergrins are involved in T cell binding to endothelial cells (Butcher and Picker, 1996). Thus, none of the adhesion molecules examined to date is a reliable marker for murine memory T cells.

The problems with these markers are further magnified when CD8<sup>+</sup> T cells are considered. Many of these markers, especially the CD45 family, show different patterns of expression between the CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets (Hou and Doherty, 1993). In addition, CD8<sup>+</sup> memory T cells exhibit increased expression of markers such as Ly6C.
(Tough et al., 1996) and CD11b (Mac-1) (McFarland et al., 1992) that are not upregulated on memory CD4+ T cells. Thus, there are clear phenotypic differences between naive and memory T cells, but none of the current markers can differentiate absolutely between the two subsets. In addition, another problem with all of these cell surface molecules is that their pattern of expression on memory T cells is similar to that on activated T cells, making them more appropriately termed activation/memory markers.

Several of the surface proteins that have altered expression patterns on memory T cells as discussed above also contribute to the distinct recirculation pathways found between naive and memory T cells. Memory T cells selectively traffic from blood to peripheral tissues, while naive T cells selectively traffic from blood to lymph nodes (Bradley and Watson, 1996). This movement of memory T cells through a broader range of tissues may also contribute to more rapid memory responses, as these cells are capable of responding to antigen in sites that are mostly devoid of professional APC. Thus, memory T cells actively patrol the distant sites of the body for the occurrence of infection, whereas naive T cell migration is relatively restricted to the lymphoid tissues that are better suited to initiate a primary immune response. Despite its fundamental importance to the long-term survival of the host, the underlying mechanisms of immunologic memory remain poorly defined.
E. CD4+ T cell response to viruses. As discussed above, CD4+ T cells are potent regulators of the immune system as a consequence of the cytokines that they are capable of producing. In contrast to the experimental bacterial and parasite models mentioned earlier, much less is known concerning the CD4+ T cell response during virus infections. CD4+ T cells provide the necessary help for B cells to secrete anti-viral antibody and may act as effector cells themselves through the secretion of anti-viral cytokines such as IFN-γ and TNF-α (Biron, 1994; Doherty et al., 1997; Zinkernagel, 1996). In several virus models CD4+ T cells are necessary to control viral infections, as mice depleted or genetically deficient in CD4+ T cells fail to control viruses such as adenovirus, herpes simplex virus (HSV), and murine gammaherpesvirus-68 (MHV-68) (Christensen et al., 1994; Doherty et al., 1997; Doherty et al., 1997; Manickan and Rouse, 1995; Thomson et al., 1996; Von Herrath and Oldstone, 1997). In addition, loss of CD4+ T cells and their impaired functions during human immunodeficiency virus (HIV) infection is associated with a loss of virus-specific CD8+ T cells and an increase in viral load as well as a greater susceptibility to other infectious agents (McMichael and Phillips, 1997; Pantaleo et al., 1997; Perelson et al., 1997).

Providing B cells with the help necessary to produce anti-viral antibodies is one of the primary roles CD4+ T cells play during virus infections. Whereas some viruses are able to directly cross-link B cell receptors and thus promote T-independent antibody responses (Szomolanyi-Tsuda and Welsh, 1996; Szomolanyi-Tsuda and Welsh, 1998),
most anti-viral antibody responses are T cell dependent and thus require CD4+ T cell help (Bachmann and Zinkernagel, 1996). Mice genetically deficient in CD4+ T cells are more susceptible to viruses in which anti-viral antibody plays an important role in resolution of the infection such as with influenza virus, Sendai virus, and vesicular stomatitis virus (VSV) (Doherty et al., 1997; Graham and Braciale, 1997). The antibody response to many viruses is dominated by secretion of antibodies of the IgG2a isotype, suggesting primarily a Th1 cell response (Doherty et al., 1997). However, the presence of Th2 cells, including the presence of antibodies of the IgG1 isotype, has been detected during several viral infections, including during the late stages of HIV infection in humans (Alonso et al., 1997; Clerici and Shearer, 1994; Cohen, 1993; Fujimura et al., 1997; Meynard et al., 1994; Mo et al., 1995; Romagnani and Maggi, 1994; Sarawar et al., 1996; Sarawar and Doherty, 1994; Wasik et al., 1997). These results suggest that the T cell cytokine responses during many virus infections may not be as polarized as the T cell responses discussed above for the bacterial and parasitic infections. However, the importance of polarized virus-specific T cell populations has been demonstrated during respiratory syncytial virus (RSV) infection. In this system, mice immunized with the F glycoprotein of RSV mount a Th1-dominated response, whereas mice immunized with the G glycoprotein of RSV mount a Th2-dominated response (Srikiatkhachorn and Braciale, 1997). Following a challenge with live RSV, mice previously immunized with the G protein show enhanced lung pathology and are more susceptible to the virus as compared to mice immunized with the F protein. Studies have demonstrated that this enhanced immunopathology is due to the
virus-specific Th2 cells induced by immunization with the G glycoprotein (Srikiatkhachorn and Braciale, 1997). In addition, studies in the influenza virus system have demonstrated that adoptive transfer of Th1 cell lines can mediate viral clearance, whereas adoptive transfer of Th2 cell lines or treatment of animals with IL-4 delay the clearance of this virus (Graham et al., 1994; Moran et al., 1996). Thus, the relative balance between a Th1 versus a Th2 cell response may also play an important role in the resolution of some virus infections.

The role of CD4+ T cells in the induction of virus-specific CD8+ T cell responses varies between experimental systems. CD8+ CTL responses to VSV, influenza virus, and HSV are significantly diminished in mice that lack CD4+ T cells (Battegay et al., 1996; Jennings et al., 1991; Tripp et al., 1995). In contrast, the CD8+ CTL response to lymphocytic choriomeningitis virus (LCMV) and ectromelia virus seem to be less dependent on CD4+ T cells (Ahmed et al., 1988; Buller et al., 1987; Doherty, 1993; Matloubian et al., 1994; Nahill and Welsh, 1993). For example, there is only a 2-fold reduction in the CD8+ cytotoxic T lymphocyte precursor (CTLp) frequency in CD4-deficient mice as compared to normal mice infected with LCMV (Von Herrath et al., 1996). The reasons why some CD8+ CTL responses are more dependent on the presence of CD4+ T cells is not clear. It has been suggested that viruses such as LCMV that induce massive CD8+ CTL responses may obviate the need for CD4+ T cell help because the frequency of responding CD8+ CTL may make enough cytokines such as IL-2 to support
the expansion and differentiation of the entire population of CD8+ CTL. The number of viral peptide-MHC complexes that an APC presents to a CD8+ CTL has also been suggested as another factor determining the requirement for CD4+ T cell help (Lezzi et al., 1998; Viola and Lanzavecchia, 1996). CD8+ CTL that are activated in a high antigen density environment may be less dependent on CD4+ T cell help. Thus, viruses that replicate more efficiently in APC, such as LCMV or ectromelia virus, will engage more TCR on the CD8+ CTL, making them less dependent on costimulation or cytokines provided by CD4+ T cells.

CD4+ T cells do play an important role in sustaining CD8+ CTL responses during chronic viral infections (Cardin et al., 1996; Matloubian et al., 1994). This has been shown most clearly in the LCMV system, in which CD8+ T cells are unable to clear a persistent LCMV infection in the absence of CD4+ T cells (Matloubian et al., 1994). Additional work in the LCMV system has demonstrated that the amount of virus necessary to clonally delete, or “exhaust,” the CD8+ CTL is significantly lower in CD4-deficient mice as compared to CD4-competent mice (Battegay et al., 1994). Such a collapse of the CD8+ T cell response has also been reported in CD4-deficient mice infected with MHV-68 (Cardin et al., 1996). In this disease, the lytic phase of the virus re-emerges several months after infection of MHC class II-deficient mice, which go on to develop a wasting disease and eventually die (Cardin et al., 1996). Recent work examining HIV-infected humans has correlated decreased CD4+ T cell responses with
elevated viral loads (Rosenberg et al., 1997). Thus, the progressive loss of virus-specific CD8+ CTL activity that occurs during HIV infection seems to parallel the decline in the CD4+ T cell numbers. The underlying mechanisms by which CD4+ T cells maintain CD8+ CTL during persistent viral infections are not well understood. The CD8+ T cells may need cytokines, such as IL-2, secreted by the CD4+ T cells in order to survive and not become exhausted in the high antigen environment (Zajac et al., 1998). Another possibility is that CD4+ T cells sustain the activation of professional APC. CD4+ T cells promote the activation of dendritic cells through the interaction of CD40-ligand on the CD4+ T cell and CD40 on the dendritic cell. Recent work has suggested that dendritic cells play a critical role in stimulating naive CD8+ T cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). Thus, during persistent virus infection, CD8+ T cells may need the CD4+ T cells to maintain the activation of the APC in order to continue the priming of naive CD8+ CTL. Interestingly, a recent report has shown that mice deficient in CD40-ligand fail to efficiently control LCMV, as high levels of virus re-emerge several months after the initial infection (Thomsen et al., 1998).

The role of CD4+ T cells in the maintenance of CD8+ T memory is less well-studied. Recent work in the LCMV system has also suggested that CD4+ T cells may be necessary to maintain virus-specific memory CD8+ CTLp (Von Herrath et al., 1996). The mechanisms by which CD4+ T cells are required to maintain memory CD8+ T cells may be similar to those discussed above for sustaining CD8+ T cell responses during
chronic viral infections. However, it would seem in this case that the secretion of cytokines such as IL-2 and IFN-γ by the CD4+ T cells may play the more important role by stimulating memory T cell proliferation and/or promoting the survival of memory CD8+ T cells.

F. Acute T cell response to LCMV. Virus infections are potent stimulators of the immune system (Ahmed and Gray, 1996; Doherty et al., 1996; Zinkernagel et al., 1996). Most examinations of these processes in viral infections have focused on CD8+ T cells, which vigorously respond to many viral infections. Infection of mice with LCMV is a well-characterized model system for studying the immune response during a virus infection (Zinkernagel, 1996). The kinetics of the CD8+ CTL response to LCMV infection varies with the infecting dose of virus as well as the age of the animal (Selin and Welsh, 1994). During a low-dose acute LCMV infection of adult C57BL/6 mice, the CD8+ T cells expand as much as 5 to 20-fold, resulting in a conversion of the CD4 to CD8 ratio from 2:1 to 1:2-3 (Selin and Welsh, 1994). The majority of these CD8+ T cells express activation markers such as CD25, CD44, and Mac-1 (McFarland et al., 1992; Razvi et al., 1995). Many of these CD8+ T cells are blast-sized by day 6 post-infection (p.i.), and by day 8 p.i., 25% of the CD8+ T cells have clearly defined cytoplasmic granules containing perforin and serine esterases (Welsh et al., 1990; Young et al., 1989). The presence of CD8+ CTL capable of lysing virus-infected targets can be easily detected.
during this time. Coincident with this activation and expansion in the CD8+ T cell number is an increase in the frequency of virus-specific CTLp as detected by limiting dilution analysis (LDA) (Assmann-Wischer et al., 1986; Selin et al., 1996). Under these experimental conditions, virus is cleared by days 7-8 p.i. and most of the activated T cells become sensitized to activation-induced cell death and die via apoptosis between days 9-14 p.i (Razvi and Welsh, 1993). Interestingly, even though there is a 10-fold reduction in the total number of CD8+ T cells during this time, the LCMV-specific CD8+ CTLp frequency per CD8+ T cell only drops 2-fold from the peak of the response and thereafter remains stable for the life of the mouse (Selin et al., 1996). This slight 2-fold reduction in the virus-specific CD8+ CTLp frequency occurs for each of the 3 immunodominant LCMV MHC class I-restricted peptides suggesting that, from the peak of the response, there is little selection in the specificity of memory T cells that are maintained into memory (Selin and Welsh, 1994). These observations have been substantiated by recent work from our laboratory showing, using complement determining region 3 length spectratyping, that the virus-induced T cell repertoire changes little from the peak of the response into memory (Lin and Welsh, 1998). A much different pattern emerges following infection of adult C57BL/6 mice with a high-dose of certain strains of LCMV (Clone 13, Docile), which have the capacity to rapidly grow to high titers in all the peripheral organs (Moskophidis et al., 1993). Under these conditions, the CD8+ T cells become activated and then clonally deleted (exhausted) under the weight of the high
antigen load, thereby preventing clearance of the virus and the establishment of CD8\(^+\) T cell memory. Thus, these mice become persistently infected with LCMV (Moskophidis et al., 1993). It is currently unclear what happens to the fate the virus-specific CD4\(^+\) T cells under these conditions of overwhelming virus infection.

Either due to a low efficiency or because a low percentage of antigen-specific cells are cytotoxic, LDA only account for a small fraction (5-10%) of the activated CD8\(^+\) T cells during the acute LCMV infection (Lau et al., 1994; Selin et al., 1996). Previous work suggested that the majority of the activated cells observed during virus infections may be due to bystander activation mediated by cytokines such as type I interferons (i.e. IFN-\(\alpha\) and IFN-\(\beta\)) that are produced in response to the viral infection (Gray, 1996; Tough et al., 1996; Tough and Sprent, 1996). However, because there is little expansion in the number of T cells not specific to the virus during infection (Zarozinski and Welsh, 1997), our laboratory concluded that the great majority of activated cells during LCMV infection is virus-specific. A similar conclusion has been reached in recent studies using MHC class I tetramers loaded with immunodominant viral peptides and using intracellular staining for IFN-\(\gamma\) following virus-peptide stimulation (Butz and Bevan, 1998; Gallimore et al., 1998; Murali-Krishna et al., 1998). These techniques can now account for nearly 70% of the CD8\(^+\) T cells that respond during an acute LCMV infection (Murali-Krishna et al., 1998). It is currently unclear if the disparity between the frequency of virus-specific CD8\(^+\) T
cells observed using these new techniques as compared to using LDA are due to the
detection of mutually exclusive populations of CD8\(^+\) T cells.

**G. T cell memory following LCMV infection.** As mentioned above, previous work
from our laboratory with several viruses has demonstrated that when the apoptotic
events reduce the T cell number after viral clearance, the virus-specific CTLp frequency
per CD8\(^+\) T cell remains remarkably high in the memory state, within 12 to 50% of the
levels seen at the peak of the acute infection (Selin et al., 1996). In addition, this virus-
specific memory CD8\(^+\) T cell response is extremely long-lasting, and, in the case of
memory CD8\(^+\) CTLp to LCMV, is maintained at steady state levels for the life-time of
the mouse (Asano and Ahmed, 1996; Lau et al., 1994; Selin et al., 1996). Most, but not
all, of the LCMV-specific memory CD8\(^+\) CTLp are found in cells bearing a
CD44\(^hi\)CD62L\(^hi\) phenotype (Razvi et al., 1995). At least some of these cells also express
IL-2 receptors and are blast-sized, suggesting that a portion of the memory CD8\(^+\) T cells
continue to cycle (Razvi et al., 1995). Moreover, our laboratory has recently
demonstrated that enriched blast-sized CD8\(^+\) T cells over a year after the infection has
cleared can still mediate direct cytotoxic activity against sensitive targets (Selin and
Welsh, 1997). These results indicate that, long after a viral infection has cleared, the
memory CD8\(^+\) T cell pool remains highly biased with virus-specific T cells, closely
reflecting the population present at its peak during infection but partially diluted with naive cells (Selin and Welsh, 1994).

Memory CD8$^+$ T cells specific for one virus can be stimulated by unrelated viruses, termed heterologous viruses. Previous studies have demonstrated that infection of LCMV-immune mice with viruses such as Pichinde virus (PV), vaccinia virus (VV), or murine cytomegalovirus (MCMV) result in a reactivation of LCMV-specific CD8$^+$ CTL (Selin et al., 1994). LDA analysis of short-term CD8$^+$ CTL clones revealed that cross-reactivity between the first and second virus accounts for at least a portion of these reactivated memory T cells (Selin et al., 1994). More recent work from our laboratory has suggested that the stable memory CD8$^+$ T cell pool may be disrupted following heterologous virus infections. Work from our laboratory has demonstrated that there is a reduction in the CD8$^+$ CTLp frequency to earlier viruses following a heterologous virus infection (Selin et al., 1996). Thus, following the resolution of an acute virus infection, the T cells from the most recent infection are selected to enter the memory T cell pool while memory T cells from earlier infections are selected against and deleted (Selin and Welsh, 1994). The mechanisms that mediate this reduction remain to be defined. It is currently unclear if CD4$^+$ T cells can also cross-react between heterologous viruses and if heterologous virus infections will disrupt the virus-specific memory CD4$^+$ T cell pool.
H. Thesis objectives. The goal of this thesis was to determine the magnitude and longevity of a CD4+ T cell response following a virus infection. Despite the importance of CD4+ T cell responses during various infections discussed above, little information exists concerning the magnitude, longevity, and stability of the CD4+ T cell response during a virus infection. The generation of the mAb CZ-1 and its initial characterization (Brutkiewicz et al., 1993; Vargas-Cortes et al., 1992) led me to first question whether this marker represented a new memory marker on CD4+ T cells. Using LCMV infection of C57BL/6 mice, one of the most well studied murine virus models, I provided evidence that CZ-1 defines a novel activation and memory marker on virus-induced murine CD4+ T cells. These studies laid the groundwork to pose additional questions, and I have taken advantage of the LCMV system to ask the following important fundamental questions:

1. Does LCMV infection elicit a cytokine response that is polarized or a mixture of Th1 and Th2 cells?

2. What is the magnitude of the CD4+ T cell response induced by LCMV infection?

3. What is the size and longevity of the memory CD4+ T cell pool that is generated following an acute virus infection?
4. Do memory CD4$^+$ T cells play any role in contributing to heterologous T cell-mediated immunity?

5. Do heterologous virus infections perturb the CD4$^+$ memory T cell pool?

6. What is the fate of the virus-specific CD4$^+$ T cells following an overwhelming virus infection?
CHAPTER II

MATERIALS AND METHODS

A. Mice. Six to eight-week old male C57BL/6J (H-2^b) and C57BL/6-Cd8a^{im1Mak} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used in this study were between 6 weeks and 2 years of age and maintained in the animal facilities at the University of Massachusetts Medical School, Worcester.

B. Viruses, protein antigens, and inoculations of mice. The LCMV Armstrong strain and the AN3739 strain of PV were propagated in baby hamster kidney cells (BHK21), as described previously (Welsh et al., 1976). The WR strain of VV was grown in L929 cells (Yang et al., 1989). MCMV, strain Smith, was obtained from the salivary glands of infected Balb/c mice (Bukowski et al., 1983). For acute virus infections, mice were inoculated intraperitoneally (i.p.) with $5 \times 10^4$ PFU’s LCMV, $4 \times 10^6$ PFU’s PV, $1 \times 10^6$ PFU’s VV, or $1 \times 10^4$ PFU’s MCMV. In order to prevent CD4^+ T cell activity against cell debris or fetal calf serum (FCS) antigens present in the virus stock, the virus preparations were either significantly diluted in phosphate buffered saline (PBS) (LCMV and MCMV) or purified over sucrose gradients and diluted in PBS (PV and VV). Mice
inoculated with a single virus were infected between 6-8 weeks of age. Immune mice used in all memory experiments were infected with virus no less than 6 weeks previously. Mice receiving multiple virus infections were immunized with a sublethal dose of one virus, rested for at least 8 weeks to allow the immune system to return to homeostasis, then challenged with the next virus. The highly disseminating LCMV Armstrong Clone 13 variant was kindly provided by Dr. M.B.A. Oldstone, Scripps Clinic and Research Foundation, La Jolla, CA. Adult mice to be persistently infected were given approximately $6 \times 10^7$ PFU's of LCMV Clone 13 intravenously (i.v.). Before injection, LCMV Clone 13 was concentrated through 30% (w/v) sucrose and diluted in PBS containing 10% normal mouse serum in order to reduce the amount of FCS antigens present in the virus stock.

For protein antigen stimulation, mice were injected i.p. with either 100 µg of keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO) in emulsified complete Freund's adjuvant (CFA, Difco Laboratories, Detroit, MI) or 100 µg ovalbumin (OVA, Sigma) in emulsified TiterMax Gold adjuvant (TMG, Sigma).

C. Preparation of cells. Mice were sacrificed by cervical dislocation, and spleens were removed aseptically. Splenic leukocytes were obtained by preparing single-cell suspensions from spleens and treating them with 0.84% NH$_4$Cl to lyse erythrocytes, as described previously (McFarland et al., 1992). Cell culture medium was either MEM
containing 10% FCS (Sigma), 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 2 mM L-glutamine, or RPMI medium (Sigma) containing FCS, penicillin, streptomycin, L-glutamine (as described above), 5 × 10^{-5} M 2-mercaptoethanol (Sigma), 0.1 μM sodium pyruvate (Gibco/BRL, Grand Island, NY), 0.1 mM nonessential amino acids (Gibco/BRL), and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

D. Antibodies and reagents. The following mAb in various conjugate combinations were used for cell surface analysis and cell sorting: anti-CD4 (clones H129.19, and RM-4-5), anti-CD8 (clone 53-6.7), anti-NK1.1 (clone PK136), anti-CD11a (clone 2D7), anti-CD25 (clone 7D4), anti-CD44 (clone IM7), anti-CD45R/B220 (clone RA3-6B2), anti-CD45RB (clone 16A), anti-CD49d (clone R1-1), anti-CD62L (clone MEL-14), and anti-CD69 (clone H1.2F3). All were purchased from Pharmingen (San Diego, CA). Tri-color conjugated anti-CD4 (clone CT-CD4) and anti-CD8 (clone CT-CD8a) were used in some multi-parameter fluorescence-activated cell sorter (FACS) experiments (both from Caltag, Burlingame, CA). L3T4 (clone GK1.5) is an anti-CD4 mAb that was used in the CD4 blocking experiments (American Type Culture Collection, Rockville, MD). The anti-CD8 (clone 2.43) used in the elutriation experiments was kindly provided by Dr. R. Fujinami, University of Utah, Salt Lake City, UT. The rat anti-mouse mAb CZ-1 (IgM) was generated in our laboratory, as described previously (Vargas-Cortes et al., 1992). For the detection of primary antibodies, the following secondary reagents were used: mouse
anti-rat γ F(ab')2-specific fluorescein isothiocyanate (FITC) from Jackson Immunoresearch Labs (West Grove, PA), avidin FITC from Becton Dickinson (San Jose, CA), and streptavidin-Red 670 from Gibco/BRL. Recombinant human IL-2 was obtained from Cellular Products (Buffalo, NY).

**E. Plaque assays.** LCMV was titrated by plaque assay on vero cell (ATCC) monolayers. Blood from LCMV-infected mice was obtained by tail-bleeding and diluted immediately 1:10 in complete RPMI and stored at -80°C. The frozen aliquots were thawed on the day of the plaque assay and used to titrate the virus on vero cells grown in 6-well plates (Costar, Cambridge, MA). Serial ten-fold dilutions of 100 μl were added to the cells in 1 ml of MEM media and incubated for 90 min at 37°C with repeated rocking. A 1:1 mixture of 1% Seakemp agarose (FMC, Rockland, ME) and EMEM (BioWhittaker, Walkersville, MD) supplemented with antibiotics and 10% FCS was overlaid onto the cells. The plates were incubated at 37°C and on day four stained with 2 ml of the above overlay mixture containing 0.1% neutral red (Sigma). LCMV plaques were counted on day 5.

**F. Centrifugal elutriation.** Spleen leukocytes were separated on the basis of size by a Beckman JE-6B centrifugal elutriation system (Beckman Instruments, Palo Alto, CA) as described previously (Biron and Welsh, 1982). At a constant rotor speed of 3200
RPM, cells were eluted at flow rates of 15, 22, 28, and 45 ml/min (Fractions 1-4). Fraction 1 contains cell debris and some erythrocytes and was discarded. Fraction 2 consists of small lymphocytes with very little cytoplasm and a mode diameter of about 6.3 μm. Fraction 3 consists of slightly larger lymphocytes with more significant cytoplasmic area, and a mode diameter of about 7.3 μm. Fraction 4 consists of large lymphocytes, macrophages, and granulocytes. Cells in fraction 4 are heterogenous in size and include virtually all of the activated blast-size lymphocytes.

G. Flow cytometry and cell sorting. For multicolor FACS analysis, approximately $1 \times 10^6$ cells were stained in staining buffer (2% FCS and 0.02% NaN$_3$ in PBS). Stained cells were resuspended in PBS, fixed with 2% paraformaldehyde, washed and resuspended in staining buffer and analyzed using a Becton Dickinson FACScan flow cytometer. Single-color controls were used in all multiparameter FACS analyses for electronic compensation settings on the flow cytometer and between 10,000 and 20,000 events were acquired from each preparation. Lymphocyte populations were first gated based on forward scatter and 90° side-scatter and then analyzed using either PC-Lysis or Cell Quest software (Becton Dickinson).

For CD4$^+$ T cell cycle analysis, splenic leukocytes from individual animals were stained with FITC-conjugated anti-CD4 (clone H129.19) from Pharmingen. After washing in staining buffer, the cells were subsequently fixed in ice-cold 95% ethanol and
then washed in PBS and stained with propidium iodide (Sigma). Approximately $1 \times 10^6$ cells were stained, and greater than 20,000 events were acquired from each preparation. The data were analyzed using Cell Quest software (Becton Dickinson).

For annexin V staining, splenocytes were stained with phycoerythrin (PE)-conjugated anti-CD4 (clone H129.19, Pharmlingen) for 20 min on ice, washed several times in PBS, and then stained with annexin V-FITC (Pharmlingen) for 15 min at room temperature in annexin V staining buffer (R&D Systems, Minneapolis, MN). Stained cells were resuspended in PBS, fixed with 2% paraformaldehyde, washed and resuspended in staining buffer and analyzed using a Becton Dickinson FACSscan flow cytometer. Approximately $1 \times 10^5$ cells were stained, and 20,000 events were acquired from each preparation. The data were analyzed using Cell Quest software (Becton Dickinson).

For sorting CZ-1$^+$ and CZ-1$^-$ subpopulations, $5 \times 10^7$ splenocytes were stained with a dilution of mAb CZ-1 for 30 min at 4°C. The cells were then incubated sequentially with mouse anti-rat $\gamma F(ab')_2$-FITC (Jackson Immunoresearch Labs), normal rat serum, and PE-conjugated anti-CD4 (Pharmlingen) with washes in between each step. The cells were resuspended in RPMI medium and sorted on a FACSTAR Plus flow cytometer from Becton-Dickinson.

For sorting CD4$^+$ and/or CD8$^+$ T cells, 1 to $2 \times 10^8$ splenocytes from a pool of 3-4 mice were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 (Pharmlingen). They were then sorted in 2-color mode at high speed on a FACSTAR Plus
flow cytometer equipped with a turbo sort package from Becton-Dickinson. When only CD4+ T cells were to be collected, the lymphocytes were first gated on CD8- cells to ensure few contaminating CD8+ T cells in the sorted CD4+ T cell populations.

For sorting CD4+ T cells expressing various activation antigens, 2 x 10^8 splenocytes from a pool of 3-4 mice were stained with PE-conjugated anti-CD4 (Pharмагen), Tricolor-conjugated anti-CD8 (Caltag), and a FITC-conjugated mAb (Pharмагen) for the activation marker of interest. The lymphocytes were first gated on CD8- cells to ensure few contaminating CD8+ T cells in the sorted CD4+ T cell populations.

H. Limiting dilution assay of virus-specific precursors. LDA to detect LCMV-specific CD4+ Th precursors (Thp) were performed based on modifications of established protocols (Ewing et al., 1995; Miller and Reiss, 1984; Topham et al., 1996). CD4+ or CD8+ T cells were sorted as described above. Sorted CD4+ and CD8+ cells were consistently >94% pure. Cultures were set up in 96-well U-bottom plates (Falcon, Lincoln Park, NJ) in a final volume of 200 μl in RPMI medium. Dilutions of sorted CD4+ T cells in 100 μl were plated as replicates of 16 or 24 microcultures with 3 x 10^4 uninfected (mock-infected with BHK21 cell supernatant) or LCMV-infected irradiated (2000 rads) peritoneal exudate cells (PEC) added in an additional 100 μl. To determine the frequency of peptide-specific CD4+ Thp, PEC were pulsed with 5 μg/ml of one of the two known LCMV MHC class II-restricted peptides, GP61-80
For determinations of KLH or OVA-specific CD4+ T cells, PEC were pulsed with 100 μg/ml of the appropriate protein antigen. The cultures were incubated at 37°C in a 95% air/5% CO₂ atmosphere. At various times after the initiation of the cultures, the plates were centrifuged at 1000 rpm for 5 min, 50 μl aliquots of the supernatants were transferred to new plates, and IL-2 activity was measured using the CTLL-2 biological assay. Briefly, the CTLL-2 cells were grown in 25-cm² flasks in complete RPMI medium supplemented with 1 U/ml of recombinant IL-2 (Cellular Products). The CTLL-2 cells were washed 3 times with complete RPMI, and 5 × 10³ CTLL-2 cells in 50 μl were added to 50 μl of the cell culture supernatants in 96-well plates and incubated for 18 h at 37°C in a 95% air/5% CO₂ atmosphere. Wells were then pulsed with 1 μCi of tritiated thymidine ([³H]TdR) (Amersham, Arlington Heights, IL) in 50 μl of complete RPMI for 6 h. The plates were harvested onto glass fiber filters, and [³H]TdR incorporation was assayed in a Wallac Betaplate scintillation counter (Wallac, Gaithersberg, MD). In 2 experiments IL-4 activity was determined by the proliferation of the IL-4 dependent cell line, CT.4S (kindly provided by Dr. W. Paul, National Institutes of Health, Bethesda, MD). Briefly, 50 μl aliquots of the supernatants were incubated for 24 h with 5 × 10³ CT.4S cells in 50 μl and then pulsed overnight with 1 μCi of [³H]TdR. Microcultures giving values in the CTLL-2 or CT.4S assay of greater than 3 standard deviations above the mean values
obtained for APC alone were scored as positive. The frequencies were corrected for the purity of the sorted CD4+ populations. Frequencies were calculated using $\chi^2$ analysis according to the method of Taswell (Taswell, 1981) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI). The frequencies reported against uninfected APC are estimated, as the curves generated from the responder dilution series often yielded linear regression lines that never crossed the 37% negative well threshold. Thus, these frequencies may reflect slight overestimates of the background response to uninfected APC. However, the response against uninfected APC was always significantly lower than that against virus-infected APC in all of the LCMV-infected mice.

I. Proliferation assays. Cultures were set up in quadruplicate in 96-well U-bottom plates (Falcon) in a final volume of 200 μl in complete RPMI medium. Sorted cells were plated at $7 \times 10^4$ cells/well in 100 μl. PEC were obtained from mice injected with thioglycollate i.p. 4 days previously and with LCMV 3 days previously. Irradiated (2000 rad) PEC ($1.5 \times 10^5$) were added to each well in a volume of 50 μl. Medium, anti-CD4 (L3T4), or IL-2 were added to the appropriate wells to a final volume of 200 μl. The plates were centrifuged briefly to pellet the cells, then incubated at 37°C in a 95% air/5% CO2 atmosphere for 4 days. After 4 days the cultures were pulsed with 1 μCi of
[\textsuperscript{3}H]TdR (Amersham) for 18 h. The cells were harvested onto glass fiber filters, and [\textsuperscript{3}H]TdR incorporation was determined by liquid scintillation counting.

**J. Intracellular cytokine staining.** Intracellular cytokine staining was performed based on modifications of recently published protocols (Murali-Krishna et al., 1998; Openshaw et al., 1995). To assess intracellular cytokine expression in cells activated nonspecifically, 2 \times 10^6 spleen cells were cultured at 37°C for 4 hr in 50 ng/ml of phorbol myristic acid (PMA, Sigma) and 500 ng/ml of ionomycin (Sigma), with either 10 \mu g/ml of brefeldin A or 2 \mu M monensin (both from Sigma) added for the final 2 hrs to enable intracellular proteins to accumulate. For antigen-specific expression of cytokines, splenocytes were cultured at 37°C for 5 hr at a concentration of 2 \times 10^6 cells/tube in a volume of 1 ml of complete RPMI medium supplemented with 10 U/ml of recombinant human IL-2 (Pharmlingen) and 10 \mu g/ml of brefeldin A or 2 \mu M monensin (both from Sigma) in the presence (5 \mu g/ml) or absence of one of the two LCMV MHC class II-restricted peptides. After 5 hr culture, the cells were harvested, washed once in staining buffer, blocked with purified anti-FcγRII/III mAb (clone 2.4G2, Pharmlingen) for 10 min on ice, and stained for surface markers for 30 min on ice. The cells were then washed in staining buffer and fixed with 2% formaldehyde in PBS for 20 min at room temperature. Following two washes with permeabilization buffer (staining buffer containing 0.5%
saponin, Sigma), the cells were resuspended in 100 μl of permeabilization buffer and incubated for 10 min at room temperature, followed by an additional 30 min incubation after the addition of a directly conjugated anti-cytokine mAb or appropriate isotype matched control mAb (Pharmlingen). The following mAb were used in various conjugate combinations: anti-IL-2 (clone JES6-5H4), anti-IL-4 (clone 11B11), anti-IL-5 (clone TRFK5), anti-IL-10 (clone JES5-16E3), anti-IFN-γ (clone XMG1.2), and anti-TNF-α (clone MP6-XT22). Approximately 2 × 10^6 cells were stained, and between 30,000 and 60,000 events were acquired from each sample. Lymphocyte populations were first gated based on forward scatter and 90° side-scatter and then analyzed using Cell Quest software (Becton Dickinson).

K. Cytokine ELISA. Unsorted splenic leukocytes or FACS-separated T cells were resuspended at a final density of 1 × 10^6 cells per ml in complete RPMI supplemented as above. Responders (100 μl) were added to 3 × 10^4 LCMV-infected irradiated (2000 rads) stimulator PEC in complete RPMI (200 μl per well total volume) and incubated at 37°C. Supernatants were harvested at 24, 48, 72, and 96 hr and stored at -80°C prior to being assayed for cytokines by enzyme-linked immunosorbent assay (ELISA). Dynatech Immulon 4 plates (Dynatech, Chantil, VA) were coated by incubation overnight at 4°C with anti-cytokine mAb diluted in PBS (50 μl per well). The plates were then washed
with PBS-0.05% Tween 20 after each of the following steps. Plates were blocked with PBS-10% FCS (200 µl per well) for 2 h at room temperature. Samples were added at 100 µl per well, and a standard curve was constructed for each plate by using eight 2-fold serial dilutions of recombinant cytokine, and the plates were again incubated overnight at 4°C prior to the addition of a biotinylated anti-cytokine mAb at 100 µl per well. After a 1 hr incubation at room temperature, 100 µl of avidin-peroxidase (1/400 dilution in PBS-10% FCS; obtained from Sigma) per well were added, and the plates were incubated at room temperature for 30 min prior to detection with 100 µl of the peroxidase substrate, 3,3'5,5'-tetramethyl-benzidine dihydrochloride (Sigma) dissolved in 0.05 M phosphate-citrate buffer, pH 5.0. The reaction was stopped with 25 µl of 2 N H₂SO₄. Plates were read at 450 nm using a THERMO MAX plate reader and analyzed using SoftMax 2.3 (both from Molecular Devices Corporation, Menlo Park, CA). The following pairs of mAb were used: anti-IL-2, JES6-1A12 and biotinylated JES6-5H4; anti-IL-4, 11B11 and biotinylated BVD6-24G2; anti-IL-5, TRFK5 and biotinylated TRFK4; anti-IL-10, JES5-2A5 and biotinylated SXC-1; anti-IFN-γ, R4-6A2 and biotinylated XMG1.2 anti-TNF-α, G281-2626 and biotinylated MP6-XT3 (all from PharMingen). The appropriate recombinant murine cytokines used as standards were also obtained from PharMingen. Limits of detection for IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α ELISA were 31 pg/ml, 125 pg/ml, 60 pg/ml, 156 pg/ml, 0.3 ng/ml, 0.3 ng/ml, respectively. IL-4 and IFN-γ
protein levels were, in some cases measured using very sensitive single-plate ELISA kits purchased from Endogen (Cambridge, MA). These kits had a limit of detection of 15 pg/ml.

L. Cytokine ELISPOT assays. Enzyme-linked immunospot assays (ELISPOT) for cytokine-secreting cells were performed based on slight modifications of established protocols (Fujihashi et al., 1993; Sarawar and Doherty, 1994). Briefly, 96-well nitrocellulose-based microtiter plates (Millititer HA; Millipore Corporation, Bedford, MA) were coated overnight at 4°C with 50 μl per well of an anti-cytokine mAb diluted in PBS. After the plates were washed with PBS, all wells were blocked with 200 μl of RPMI containing 10% FCS for 2 h at 37°C. After washing with PBS, the lymphocyte populations (either splenic leukocytes or FACS-separated T cell populations) were then added to the wells (1 × 10^3 to 1.3 × 10^4 or from 1 × 10^5 to 7.8 × 10^2 cells per well for responders with 3 × 10^4 LCMV-infected irradiated (2000 rads) PEC as stimulators) in RPMI-10% FCS (200 μl per well total volume) and incubated for 20 h at 37°C. After the wells were washed in PBS-Tween, a biotinylated anti-cytokine mAb was added, diluted in PBS containing 10% FCS (100 μl per well), and incubated overnight at 4°C. Plates were washed in PBS-Tween, and 100 μl of an anti-biotin mAb conjugated with peroxidase (1/250 dilution in PBS-10% FCS; Jackson Immunoresearch Labs) per well was added
followed by another overnight incubation at 4°C. Spots representing individual cytokine-secreting cells were visualized by developing with the substrate 3-amino-9-ethylcarbazole (Sigma) and counted using an Olympus SZ-STS Stereozoom microscope (Lake Success, NY). All assays were performed in triplicate. Mean numbers of cytokine-secreting cells were calculated from the triplicate assays. The following pairs of mAb were used in the ELISPOT assays: anti-IL-4, BVD4-1D11 and biotinylated BVD6-24G2; anti-IFN-γ, R4-6A2 and biotinylated XMG1.2. The mAb were used at a concentration of 2 µg/ml, with the exception of R4-6A2, which was used at 10 µg/ml. All of the mAb were obtained from Pharmingen.

M. Statistical analysis. CD4⁺ T helpers frequencies were calculated using $\chi^2$ analysis according to the method of Taswell (Taswell, 1981) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI). All other data, where appropriate, were analyzed by Student’s t-test for each individual experiment using Microsoft’s Excel software (Microsoft Corporation, Redmond, WA). Statistical significance was established at a $p$ value of $<0.05$. 
CD4+ T cells play a central role in the immune system. They can provide help for B cells to produce antibodies, can provide help for CD8+ T cell responses, can activate macrophages to become more bactericidal, and can act as effector cells themselves (Biron, 1994; Doherty et al., 1997; Zinkernagel, 1996). Most of the in vivo studies examining CD4+ T cells have been performed using well-defined protein antigens that elicit strong CD4+ T cell responses (Swain et al., 1996). Much less work has been performed examining the CD4+ T cell response during viral infection. LCMV infection of mice is one of the most well studied experimental viral systems, yet surprisingly little information exists concerning the magnitude of CD4+ T cell response during this model virus infection. As mentioned in the introduction, activated T cells modulate the expression of several cell surface molecules. In this chapter, I examined the cell surface phenotype of CD4+ T cells during an acute LCMV infection into memory. I will show that a larger proportion of the CD4+ T cells express activation markers during the acute LCMV infection as compared to naive mice. In addition, I will provide evidence that CZ-1, a novel mAb produced in our
laboratory (Brutkiewicz et al., 1993; Vargas-Cortes et al., 1992), represents a new activation and memory marker for mouse CD4\(^+\) T cells.

Besides cell surface phenotype, CD4\(^+\) T cells may also be characterized by the cytokines they produce (Mosmann et al., 1986; Mosmann and Coffman, 1989). Little quantitative information is known about the frequencies and types of cytokine-secreting CD4\(^+\) T cells which develop after experimental viral infections. The production of IFN-\(\gamma\) may be used to detect Th1 cells whereas the production of IL-4 may be used to detect Th2 cells (Mosmann and Coffman, 1989). Two recent reports have detected both IFN-\(\gamma\)- and IL-4-secreting cells following LCMV infection, suggesting that a mixture of both Th1 and Th2 cells are induced (Su et al., 1998; Whitmire et al., 1998). In this chapter I will show, using ELISPOT assays to examine cytokine production at the single cell level, that LCMV infection induces primarily a Th1 response characterized by an increased frequency of IFN-\(\gamma\)-producing CD4\(^+\) and CD8\(^+\) T cells. In addition, I will provide evidence that at least a portion of the Th2 responses that have been recently reported (Su et al., 1998; Whitmire et al., 1998) may be due to FCS or cellular antigens present in the virus stocks and not induced by the virus.

A. T cell distribution and activation phenotype of CD4\(^+\) T cells during the acute LCMV infection and on into memory. Figure III-1 shows the percentage of CD4\(^+\) and CD8\(^+\) T cells in the spleen of C57BL/6 mice acutely infected with LCMV. Also shown
Figure III-1. Phenotype of splenic cell populations during an acute LCMV infection of C57BL/6 mice. Cell numbers, expressed as cell counts per spleen (●), and cell phenotypes, expressed as percentages of total splenic leukocytes, were determined by FACS analysis, as described in the Materials and Methods. Means ± standard deviations for time points from 2 separate experiments with 4 individual mice per experiment are shown (n=8 per group).
in Figure III-1 is the 2-fold increase in the total leukocyte number in the spleen that occurs during the acute LCMV infection. The increase in the percentage of CD8+ T cells combined with the increase in the total number of cells results in a massive 5- to 20-fold expansion in the total number of CD8+ T cells in the spleen during LCMV infection (McFarland et al., 1992). In contrast, there is only a modest increase in the total number of CD4+ T cells in the spleen, as the percentage of CD4+ T cells has decreased at the peak in the leukocyte number (Fig. III-1). Although the total CD4+ T cell number remains relatively stable, further analyses revealed evidence of CD4+ T cell activation as shown by blastogenesis and activation antigen expression. The mean percentage of blast-sized CD4+ T cells from 4 individual mice per group were as follows: day 0: 8; day 3: 9; day 5: 16; day 7: 25; day 9: 21; day 11: 19; day 15: 11. Analysis of DNA content by propidium iodide staining revealed an increase in the percentage of CD4+ T cells in the cell cycle. The mean percentages of CD4+ T cells in G2+M or S phase from 6 individual mice per group were as follows: day 0: 2±1; day 7: 7±2, day 9: 6±2, day 11: 5±1; 7 mo: 2±1; 11 mo: 2±1; 19 mo: 2±1. Figure III-2 shows the distribution of CD4+ T cells following an acute infection with LCMV with regard to the antigens CD44 (Pgp-1), a marker whose cell surface expression increases on activated T cells (Butterfield et al., 1989), and CD62L (MEL-14), a molecule whose cell surface expression decreases on activated T cells (Bradley et al., 1993). Table III-1 shows that there is an increase in the mean fluorescence intensity (MFI) of CD44 on gated CD4+ T cells, and as can be seen in Figure III-2, this
Figure III-2. Expression of CD62L and CD44 on CD4⁺ T cells during LCMV infection. Spleen cells were from mice either uninfected (day 0) or infected with LCMV for 5, 10 or 60 days. Splenocytes were stained with anti-CD4 and either anti-CD62L (top) or anti-CD44 (bottom) mAb. Numbers in the quadrants represent the percentage of cells that fall within that quadrant.
Table III-1. Mean Fluorescence Intensity of Activation Markers on CD4\(^+\) T Cells During the Acute LCMV Infection and on into Memory\(^a\)

<table>
<thead>
<tr>
<th>Day P.I.</th>
<th>CD62L</th>
<th>CD44</th>
<th>CD45RB</th>
<th>CD49d</th>
<th>LFA-1</th>
<th>CD25</th>
<th>CD69</th>
<th>CZ-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>50±9</td>
<td>281±20</td>
<td>133±15</td>
<td>28±15</td>
<td>93±7</td>
<td>14±2</td>
<td>14±1</td>
<td>22±3</td>
</tr>
<tr>
<td>Day 5</td>
<td>42±4</td>
<td>261±35</td>
<td>196±9(^*)</td>
<td>46±7(^*)</td>
<td>109±7(^*)</td>
<td>25±5(^*)</td>
<td>40±6(^*)</td>
<td>32±4(^*)</td>
</tr>
<tr>
<td>Day 10</td>
<td>36±3(^*)</td>
<td>517±88(^*)</td>
<td>183±22(^*)</td>
<td>54±14(^*)</td>
<td>140±17(^*)</td>
<td>15±1</td>
<td>17±4</td>
<td>39±4(^*)</td>
</tr>
<tr>
<td>Day 15</td>
<td>48±5</td>
<td>438±40(^*)</td>
<td>163±15(^*)</td>
<td>49±5(^*)</td>
<td>135±10(^*)</td>
<td>19±1(^*)</td>
<td>17±1</td>
<td>21±2</td>
</tr>
<tr>
<td>Day 60</td>
<td>50±1</td>
<td>290±33</td>
<td>132±10</td>
<td>27±5</td>
<td>96±6</td>
<td>13±1</td>
<td>14±2</td>
<td>31±4</td>
</tr>
<tr>
<td>Day 10 BHK(^b)</td>
<td>48±10</td>
<td>277±14</td>
<td>137±9</td>
<td>25±4</td>
<td>88±4</td>
<td>13±1</td>
<td>13±1</td>
<td>23±2</td>
</tr>
<tr>
<td>Day 60 BHK(^b)</td>
<td>52±3</td>
<td>278±17</td>
<td>153±18</td>
<td>28±2</td>
<td>92±3</td>
<td>13±2</td>
<td>14±1</td>
<td>23±2</td>
</tr>
</tbody>
</table>

\(^a\)The mean fluorescence intensity (MFI) indicates the channel number in a linear scale, which corresponds to the mean of the fluorescence intensities obtained for a particular mAb. Means ± standard deviations of 4 individual mice from 1 of 2 similar experiments are shown.

\(^b\)Mice were injected with BHK21 cell supernatant as a control.

\(^*\)Indicates a significant difference as compared to Day 0, \(p<0.05\).
increase in the MFI is mainly due to an increase in the relative proportion of CD4⁺ T cells expressing a high level of CD44 rather than all of the CD4⁺ T cells staining more highly with CD44. Likewise, Table III-I shows that there is a decrease in the MFI of CD62L on gated CD4⁺ T cells, and as can be seen in Figure III-2, this decrease in the MFI seems to be due to an increase in the relative proportion of CD4⁺ T cells expressing a low level of CD62L. A similar pattern emerged when the cell surface expressions of CD25 (IL-2 receptor) and CD69 (very early activation antigen) were examined on CD4⁺ T cells following an acute LCMV infection (Fig. III-3). However, the highest proportion of CD4⁺ T cells expressing high amounts of these markers was at day 5 p.i. (p<0.05), in contrast to CD62L and CD44 discussed above. Figure III-4 shows a more detailed time course of the cell surface expression of CD62L, CD44, and CD69 on gated CD4⁺ T cells. Many of the CD4⁺ T cells stained CD62LloCD44hi 7-11 days p.i., characteristic of an activated T cell phenotype (McHeyzer-Williams et al., 1996; Swain and Bradley, 1992). The peak in cell surface expression of the activation marker CD44 (day 9 MFI: 259; day 11 MFI: 252) at days 9-11 p.i. correlated with the lowest cell surface expression of CD62L (day 9 MFI: 34; day 11 MFI: 35), a molecule whose cell surface expression decreases on activated T cells (Bradley et al., 1993). In contrast to CD62L and CD44, CD4⁺ T cell surface expression of the very early activation antigen CD69 peaked much earlier at 3 days p.i. (MFI: 41) and declined thereafter. More detailed analysis of CD69
Figure III-3. Expression of CD25 and CD69 on CD4⁺ T cells during LCMV infection. Spleen cells were from mice either uninfected (day 0) or infected with LCMV for 5, 10 or 60 days. Splenocytes were stained with anti-CD4 and either anti-CD25 (top) or anti-CD69 (bottom) mAb. Numbers in the quadrants represent the percentage of cells that fall within that quadrant.
Figure III-4. Expression of CD44, CD62L, and CD69 on CD4$^+$ T cells during LCMV infection. Spleen cells from mice either uninfected (day 0) or infected with LCMV for 3, 5, 7, 9, 11, or 15 days. Splenocytes were stained with anti-CD4 and either anti-CD44, anti-CD62L, or anti-CD69 mAb. A CD4 gate was applied, and the histograms represent the cell surface expression of CD44, CD62L, or CD69 on the gated CD4$^+$ T cells. Data are representative of 2 separate experiments with 4 individual mice per experiment ($n$=8 per group).
expression on CD4+ T cells early after LCMV infection revealed that expression of this marker peaked at days 2-3 p.i. (data not shown).

In addition, I examined the cell surface expression of several other activation markers and found increases in the MFI of CZ-1 (a sialated form of CD45RB), CD25, and CD69 as well as of the adhesion molecules CD49d (VLA-4) and LFA-1 (CD11a) on gated CD4+ T cells after LCMV infection (Table III-I). CD45RB cell surface expression has been shown to increase on effector CD4+ T cells (Bradley et al., 1993). In agreement with this, I observed an increase in the cell surface expression of CD45RB on CD4+ T cells at day 10 p.i. with LCMV. Interestingly, while CD45RB expression increases on activated cells and then decreases on memory CD4+ T cells, the antigen CZ-1, a sialic acid-dependent CD45RB-associated epitope, is bright on activated cells and remains high on memory CD4+ T cells. This is reflected in the higher expression of CZ-1 vs. CD45RB on memory CD4+ T cell populations (day 60 p.i., Table III-I). Thus, LCMV infection induces on CD4+ T cells an activated phenotype that peaks by days 9-11 p.i.

B. Characterization of CZ-1 as a novel CD4+ T cell activation marker. The above experiments revealed the modulation of several activation markers on CD4+ T cells during an acute infection with LCMV. The level of cell surface CD45RB expression has been used to distinguish between naive and memory CD4+ T cells (Bradley et al., 1993; Vitetta et al., 1991). CD4+ T cells stain heterogenously with anti-CD45RB mAb (Ernst et
al., 1990). Because mAb CZ-1 defines a sialic acid-dependent CD45RB-associated epitope (Brutkiewicz et al., 1993), I compared the cell surface expression of the CZ-1 antigen with that of CD45RB on splenocytes following an acute LCMV infection. As expected, the staining pattern of CD4+ T cells was heterogeneous with respect to CD45RB expression. Figure III-5 shows that mAb CZ-1 stained the CD4+ T cells from uninfected mice at a lower intensity than did the anti-CD45RB mAb, with MFI of 115 and 259, respectively. In contrast, mAb CZ-1 stained the CD4- cells at a higher intensity than did the anti-CD45RB mAb (MFI of 1019 and 375, respectively). CD4+ T cells stained more brightly for the CZ-1 antigen at day 6 p.i. (MFI of 206), as compared to uninfected mice (MFI of 115). In the immune mice (day 42 p.i.), mAb CZ-1 stained the CD4+ T cells with a slightly lower intensity (MFI of 191) overall, but some CD4+ T cells stained brightly for the CZ-1 epitope.

Since CZ-1 and CD45RB showed slightly different staining patterns, I determined the amount of coexpression of these two molecules on CD4-gated splenocytes before and during an acute infection with LCMV. Uninfected and day 6 p.i. splenocytes were stained with anti-CD4, anti-CD45RB, and CZ-1 (Fig. III-6). In the uninfected mice, both CZ-1 and anti-CD45RB stained CD4-gated splenocytes heterogeneously, with most cells being CZ-1lo and CD45RBhi. However, at day 6 p.i., a subpopulation of cells stained CD45RBlo and CZ-1hi. Thus, during an acute infection with LCMV, there is a subpopulation of CD4+ T cells that stain CD45RBloCZ-1hi.
Figure III-5. Expression of CZ-1 and CD45RB on splenocytes during LCMV infection. Spleen cells from mice either uninfected (day 0) or infected with LCMV for 6 or 42 days were stained with CZ-1 and CD4 mAb. Numbers represent the mean fluorescence intensity (x-axis) of the CD4+ and CD4- populations.
Figure III-6. Coexpression of CZ-1 and CD45RB on splenocytes during an acute LCMV infection. Spleen cells from mice either uninfected (day 0) or LCMV-infected (day 6) were stained with CD4, CD45RB, and CZ-1 mAb. A CD4 gate was applied and the dot plots show CD45RB versus CZ-1.
To further analyze the phenotype of the activated CD4+ T cells during an acute infection with LCMV, I stained splenocytes with anti-CD4 and mAb specific for various cell surface markers and examined their density of expression in relation to cell size (as determined by forward light scatter). Figure III-7 (upper panel) shows that the blast-sized CD4+ T cells at day 6 p.i. with LCMV were CZ-1hi. In contrast, the majority of the blast-sized cells were CD45RBlo (Fig. III-7 lower panel). Figure III-8 shows that the blast-sized CD4+ T cells during the acute infection were CD44hi and CD62L-. Three-color FACS analysis was performed (CD4 and CZ-1 vs. CD44 or CD62L) to further compare the cell surface expression of the CZ-1 antigen with the CD44 and CD62L molecules. The population of cells with the highest CD44 expression at day 6 p.i. were also CZ-1hi (Fig. III-9 upper panel). In addition, Figure III-9 (lower panel) shows that those cells with low CD62L expression include mostly CZ-1hi cells. Thus, the phenotype of the blast-sized cells during an acute infection with LCMV is CZ-1hiCD45RBloCD44hiCD62L-.

C. CZ-1 also serves as a memory marker for virus-induced CD4+ T cells. Because some activation molecules continue to be expressed on memory cells, I questioned whether LCMV-specific memory CD4+ T cells expressed elevated levels of CZ-1. Figure III-10 shows the two-color FACS analysis of CZ-1 antigen expression on CD4+ T cells in an LCMV-immune spleen six weeks p.i. The bimodal staining pattern
Figure III-7. Surface phenotype of blast-sized cells during an LCMV infection. Splenocytes from mice either uninfected (day 0) or during an acute (day 6) infection with LCMV were stained for CD4 and either CZ-1 or CD45RB. A CD4 gate was applied, and the dot plots show either CZ-1 or CD45RB versus cell size, as measured by forward light scatter.
Figure III-8. Expression of CD44 and CD62L on blast-sized CD4+ T cells during an acute LCMV infection. Splenocytes from mice either uninfected (day 0) or infected (day 6) with LCMV were stained for CD4 and either CD44 or CD62L. A CD4 gate was applied, and the dot plots show either CD44 or CD62L versus cell size, as measured by forward light scatter.
Figure III-9. Comparison of CD44 and CD62L expression with the CZ-1 antigen. Spleen cells from mice either uninfected (day 0) or LCMV-infected (day 6) were stained with CD4, CZ-1, and either CD44 or CD62L mAb. A CD4 gate was applied and the dot plots show either CD44 or CD62L versus CZ-1.
Figure III-10. Expression of CZ-1 on splenocytes from an LCMV-immune animal. Splenocytes were stained as described in the Materials and Methods and sorted into two populations based on CD4 and CZ-1 expression. The R1 gate representing the CD4⁺CZ-1⁻ population and the R2 gate representing the CD4⁺CZ-1⁺ population are shown. Numbers in the quadrants of the post-sort profiles represent the percentages of cells per quadrant.
was similar to that found in Figure III-5. I sorted splenocytes from LCMV-immune mice into CD4+CZ-1+ and CD4+CZ-1- populations (Fig. III-10) and determined their ability to proliferate in response to LCMV-infected PEC in vitro (Fig. III-11). In eight independent experiments, I found that all of the virus-specific memory was in the CD4+CZ-1+ compartment. Furthermore, treatment of unsorted splenocytes from an LCMV-immune mouse with mAb CZ-1 and complement abolished this proliferative response to virus-infected PEC (data not shown).

Because mAb CZ-1 also stains CD8+ T cells (Vargas-Cortes et al., 1992), which represented a very small contaminant (<1%, see Fig. III-10) in the sorted populations, I wanted to ensure that it was the CD4+ and not the CD8+ T cells that were responding in the proliferation assays. As shown in Figure III-11, treatment with anti-CD4 (Chance et al., 1994) blocked all of virus-specific proliferation from the CD4+ CZ-1+ population. Furthermore, the purity of the expanded CD4+ CZ-1+ culture was verified by staining cells pooled from several wells of the proliferation assay with anti-CD4 and anti-CD8 mAb. The cultures stained >92% for CD4+ cells, with CD8+ cells accounting for <1% of the population (Fig. III-12).

The lack of any memory in the CD4+ CZ-1- population could have been the result of an increased requirement for T cell growth factors such as IL-2, as these cells could have been in a lower activation state than the CD4+ CZ-1+ cells. However, the CD4+ CZ-1- population did not show any significant proliferation with the addition of exogenous IL-2.
Figure III-11. LCMV-specific memory CD4+ T cells express the CZ-1 antigen. The results from one of several experiments are shown in which splenocytes were FACS-purified into CD4+CZ-1- and CD4+CZ-1+ and then assayed for [3H]TdR incorporation in response to stimulation with LCMV-infected PEC. Some wells were incubated with anti-CD4 mAb or various concentrations of IL-2. Only the highest IL-2 concentration used, 13.5 U/ml, is shown. There was no proliferative response from the CD4+CZ-1- population over the range of IL-2 concentrations tested (0.5-13.5 U/ml). Error bars represent the standard deviations of quadruplicate cultures.
Figure III-12. Predominantly CD4\(^+\) T cells grow out in the proliferation cultures. A pool from 4 wells of the expanded CD4\(^+\)CZ-1\(^+\) culture was stained with CD4 and CD8 mAb. The dot plots show CD4 versus CD8. Numbers in the quadrants represent the percentage of cells that fall within that quadrant.
in the presence of virus-infected APC (Fig. III-11). Previous work from our laboratory has shown that whereas CZ-1\(^+\) T cells do not respond to IL-2 in the absence of antigen, they do proliferate in response to concanavalin A or anti-CD3. In addition, our laboratory has shown that the CZ-1 antigen is expressed on CD4\(^+\) T cells after activation with anti-CD3 (Brutkiewicz et al., 1993; Vargas-Cortes et al., 1992).

Some investigators define memory CD4\(^+\) T cells as small resting cells and distinguish them from activated cycling cells that may persist for a time after antigen exposure (Croft et al., 1994). To ensure that such chronically activated CD4\(^+\)CZ-1\(^+\) T cells were not responsible for all of the virus-specific proliferation measured in the memory T cell proliferation assays, I examined the size of the CD4\(^+\)CZ-1\(^+\) T cells in LCMV-immune mice. Figure III-13 shows that there was not a significant increase in blast-sized CD4\(^+\)CZ-1\(^+\) T cells in LCMV-immune mice as compared to uninfected control animals (see Fig. III-7 upper panel). The blast-size CD4\(^+\) T cells in the LCMV-immune mice were few in number and considerably less than the frequency of blast-sized CD8\(^+\) T cells, reported previously by our laboratory (Razvi et al., 1995). To determine whether the small CD4\(^+\)CZ-1\(^+\) memory T cells could proliferate when stimulated \textit{in vitro} with LCMV-infected PEC, I utilized an elutriator centrifuge to separate splenic leukocytes based on cell size. The cells were then depleted of CD8\(^+\) T cells using anti-CD8 mAb and complement and stimulated against LCMV-infected PEC in the presence of 1 U/ml IL-2. Proliferation was seen in cells from Fraction 4, which contains nearly all of the blast-sized
Figure III-13. Small CD4⁺ T cells in LCMV-immune mice express the CZ-1 antigen. Splenocytes from LCMV-immune (day 42) mice were stained for CD4 and CZ-1. A CD4 gate was applied, and the dot plots show CZ-1 versus cell size, as measured by forward light scatter.
lymphocytes, as well as contaminating small cells. However, significant proliferation was also observed in cells from Fractions 2 and 3, which contain very little contamination from larger blast-sized cells (F2: 15,545±2,767 cpm; F3: 35,785±30,012 cpm; F4: 29,677±2,128 cpm) (Biron and Welsh, 1982). The total cell numbers per fraction were higher in Fractions 2 and 3 than they were in Fraction 4 (F2=3.1 x 10^7 cells, F3=4.7 x 10^7 cells, F4=2.3 x 10^7 cells), indicating that most of the memory cells were small lymphocytes. In a second experiment, mAb to CZ-1 and complement was used to treat elutriated Fraction 2 cells, and this caused a 71% reduction in their proliferative response (2,709±414 cpm vs. 771±475 cpm). Finally, Fraction 2 cells were sorted by flow cytometry into small CD4^+CZ-1^+ and small CD4^+CZ-1^- populations and placed into a proliferation assay at 7 x 10^4 cells per well. Virtually all of the proliferation in response to LCMV-infected PEC was in the CZ-1^+ population (CD4^+CZ-1^+: 2,690±3,350 cpm; CD4^+CZ-1^-: 33±17 cpm). These experiments indicate that even the very small lymphocytes contain LCMV-specific memory T cells and that the CZ-1^+ subpopulation contains virtually all of that memory.

**D. Cytokine secretion during the acute LCMV infection.** The above experiments show that CD4^+ T cells expressing an activated cell surface phenotype are induced following an acute LCMV infection. Since one of the primary effector functions of CD4^+ T cells is the secretion of various cytokines, I utilized the ELISPOT assay to assess IFN-
γ and IL-4 production at the single-cell level in C57BL/6 mice acutely infected with LCMV. During the course of my early studies using the ELISPOT assay, it became apparent that a portion of the cytokine response being measured was due to a memory T cell response to FCS or cellular antigens present in our virus stocks. Thus, when I infected mice with a LCMV stock that had been grown in BHK21 cells in medium containing 10% FCS, a significant FCS-specific CD4+ T cell response would be generated in addition to the virus-specific T cell response. These FCS antigen-specific CD4+ T cells would produce both IFN-γ and IL-4 when placed in the ELISPOT assay. Table III-2 demonstrates these findings by showing that once the virus stock is diluted in PBS, there is a significant reduction in the frequency of cytokine-producing cells. This suggests that most of the cytokine-producing cells previously detected were specific to FCS antigens that would be constantly present in these in vitro assays. The addition of LCMV-infected PEC to the assay resulted in a significant increase in the number of virus-specific IFN-γ-producing cells, with only a slight increase in the number of IL-4-producing cells. Even though the addition of the LCMV-infected PEC as APC does increase the number of IL-4-producing cells, the proportional increase is not nearly as high as the increase that is observed for IFN-γ, and it is less than the number detected following infection with an undiluted virus. This observation, is important to note due to recent studies that have reported, I believe erroneously, significantly elevated frequencies of IL-4-producing cells following LCMV-infection (Su et al., 1998; Whitmire et al., 1998). Because of these
Table III-2. Frequency of IFN-γ and IL-4-Secreting Cells on Day 7 Post-Infection with LCMV

<table>
<thead>
<tr>
<th></th>
<th>In vivo priming</th>
<th>INF-γ+ Cells per 1 x 10^6</th>
<th>IL-4+ Cells per 1 x 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMV Day 7 Undiluted</td>
<td></td>
<td>155 ± 122</td>
<td>86 ± 26</td>
</tr>
<tr>
<td>LCMV Day 7 Dil. 1:100 in PBS</td>
<td></td>
<td>11 ± 6</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>LCMV Day 7 Dil. 1:100 in PBS</td>
<td></td>
<td>&gt;1600</td>
<td>52 ± 19</td>
</tr>
<tr>
<td></td>
<td>Plus LCMV APC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHK Sup. Day 7 Dil. 1:100 in PBS</td>
<td></td>
<td>0 ± 0</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>BHK Sup. Day 7 Dil. 1:100 in PBS</td>
<td>Plus LCMV APC</td>
<td>8 ± 6</td>
<td>7 ± 9</td>
</tr>
</tbody>
</table>

ELISPOT assays were performed as described in the Materials and Methods on spleen cells from LCMV-infected mice. Means ± standard deviations for 4 individual mice are shown.
findings, all of the experiments presented hereafter were performed with viruses either highly diluted in PBS or purified over sucrose gradients.

Figure III-14 shows the frequency of cytokine-producing cells in the spleen of C57BL/6 mice infected with LCMV that has been diluted in PBS before injection. Uninfected mice (day 0) had no detectable IFN-γ- or IL-4-secreting cells (hereafter referred to as IFN-γ+ and IL-4+ cells) in the spleen. Following an infection with LCMV, the frequency of IFN-γ+ cells per spleen leukocyte started to increase by day 5 p.i. and reached its peak by day 9 p.i. On a per spleen basis, the peak in the total number of IFN-γ+ cells was even more pronounced at days 7-11 p.i., because the number of cells in the spleen had nearly doubled (Fig. III-1). By day 15 p.i. the frequency of IFN-γ+ cells had started to decline. The decline in the frequency of IFN-γ+ cells after day 7-11 p.i. coincides with the clearance of the virus and the decline of the CD8+ CTL response (Selin and Welsh, 1994). In contrast to IFN-γ, few IL-4+ cells could be detected in the spleen.

I performed several additional experiments to determine if any significant virus-specific IL-4 production could be detected following LCMV infection. Cytokine production was first analyzed for protein production in culture supernatants from cells that had been restimulated in vitro with virus-infected irradiated APC. In a single experiment with four individual mice, splenocytes from LCMV-infected mice produced a mean of 4,762 pg/ml of IFN-γ at day 7 p.i. and 23,540 pg/ml of IFN-γ at day 15 p.i., with
Day 0
Day 3
Day 5
Day 7
Day 9
Day 11
Day 15
Day 7 BHK
Day 15 BHK

Number of Cytokine-Secreting Cells Per $10^5$ Splenic Leukocytes

Figure III-14. Frequency of cytokine-producing cells in LCMV-infected C57BL/6 mice. ELISPOT assays were performed for IFN-γ and IL-4 on freshly isolated splenocytes taken 3 to 15 days after i.p. infection with LCMV or 7 and 15 days after i.p. injection of a BHK21 supernatant control. The day 0 time point represents uninfected mice. Means ± standard deviations for time points from two separate experiments with two individual mice per experiment are shown ($n=4$ per group).
no IL-4 detected at either time point. In another experiment, conditioned media was prepared from splenocytes at day 9 p.i. followed by assay of the supernatant for cytokine production by ELISA. In a single experiment using 2 individual mice, no IFN-\(\gamma\) or IL-4 could be detected in the absence of antigen stimulation. However, with the addition of 1 \(\mu\)g/ml of one of two recently described LCMV-encoded MHC class II-restricted (I-A\(^b\)) CD4 peptides (Oxenius et al., 1995), production of high levels of IFN-\(\gamma\) and very low levels of IL-4 protein became detectable (day 9 GP61-80 peptide stimulated: 1,292 pg/ml IFN-\(\gamma\); 33 pg/ml IL-4). No IFN-\(\gamma\) or IL-4 could be detected using the other LCMV MHC class II-restricted CD4 peptide, NP309-328. These experiments show that very little, if any, virus-specific IL-4 production can be detected following LCMV infection, even after stimulation with either of the two known LCMV MHC class II-restricted peptides. Thus, these results suggest that LCMV infection induces primarily a Th1 response.

**E. CD4\(^+\) T cells produce more IFN-\(\gamma\) than CD8\(^+\) T cells.** CD4\(^+\) and CD8\(^+\) T cells from the spleens of mice that had been acutely infected with LCMV were purified by cell sorting in order to determine if virus-specific CD4\(^+\) T cells contributed to the secretion of IFN-\(\gamma\) in the ELISPOT assays described above. Table III-3, which presents the results from 2 to 3 separate experiments per time point, shows that CD8\(^+\) T cells made up the majority of IFN-\(\gamma\)-secreting cells during the acute LCMV infection. However, there were
Table III-3. Frequency of IFN-γ-Secreting Cells and the Quantity of IFN-γ Secreted into the Supernatant by Sorted CD4⁺ and CD8⁺ T Cells During the Acute LCMV Infection

<table>
<thead>
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<tbody>
<tr>
<td></td>
<td></td>
<td>per 1 x 10⁵ (pg/ml)</td>
<td>per 1 x 10⁵ (pg/ml)</td>
<td>per 1 x 10⁵ (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 9</td>
<td>1</td>
<td>683</td>
<td>20,692</td>
<td>159</td>
<td>ND</td>
<td>1,136</td>
<td>2,678</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>243</td>
<td>ND</td>
<td>24</td>
<td>ND</td>
<td>165</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1,133</td>
<td>13,656</td>
<td>200</td>
<td>ND</td>
<td>2,645</td>
<td>5,176</td>
</tr>
<tr>
<td>Day 10</td>
<td>1</td>
<td>605</td>
<td>14,386</td>
<td>117</td>
<td>6,895</td>
<td>1,536</td>
<td>9,595</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1,205</td>
<td>5,775</td>
<td>106</td>
<td>ND</td>
<td>1,877</td>
<td>3,353</td>
</tr>
<tr>
<td>Day 11</td>
<td>1</td>
<td>771</td>
<td>ND</td>
<td>205</td>
<td>ND</td>
<td>613</td>
<td>3,408</td>
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<td></td>
<td>2</td>
<td>355</td>
<td>ND</td>
<td>95</td>
<td>ND</td>
<td>587</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1,077</td>
<td>6,647</td>
<td>35</td>
<td>17,719</td>
<td>1,269</td>
<td>4,437</td>
</tr>
<tr>
<td>Day 15</td>
<td>1</td>
<td>253</td>
<td>39,390</td>
<td>28</td>
<td>9,509</td>
<td>229</td>
<td>24,683</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>121</td>
<td>10,022</td>
<td>25</td>
<td>8,357</td>
<td>169</td>
<td>4,519</td>
</tr>
<tr>
<td>Day 180</td>
<td>1</td>
<td>23</td>
<td>3,626</td>
<td>0</td>
<td>&lt;156</td>
<td>73</td>
<td>920</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>59</td>
<td>ND</td>
</tr>
</tbody>
</table>
ELISPOT and ELISA assays were performed as described in the Materials and Methods on pooled spleen cells (3-4 mice) from LCMV-infected mice that were separated by FACS and restimulated with virus in vitro. The results shown are for the maximum level of cytokine that could be detected by ELISA. No IL-4 was detected by ELISPOT assay or ELISA and no IL-5 or IL-10 was detected by ELISA in any of these cultures.

ND, not done.
detectable frequencies of LCMV-specific CD4^+ T cells secreting IFN-\( \gamma \). The ELISPOT assay detects secreted cytokine molecules in the immediate vicinity of the cell from which they are derived, with each spot representing a footprint of the original cytokine-secreting cell (Czerkinsky et al., 1983). I noticed in these assays that, while there were on average 11-fold more CD8^+ than CD4^+ T cells secreting IFN-\( \gamma \), the CD4^+ "spots" in the assays were consistently larger. This suggested that the CD4^+ T cells may be making more IFN-\( \gamma \) on a per cell basis than the CD8^+ T cells. Previous work with in vitro systems has shown that CD4^+ T cells can secrete higher levels of cytokines than similarly stimulated CD8^+ T cells (Carter and Dutton, 1996). To examine if this were the case during LCMV infection, I FACS-purified CD4^+ and CD8^+ T cells at various times p.i., restimulated them in vitro with virus, and performed ELISA assays on the culture supernatants. Table III-3 shows that CD4^+ T cells secreted as much IFN-\( \gamma \) protein into the cell culture supernatant as CD8^+ T cells, even though there were 11-fold more CD8^+ T cells secreting IFN-\( \gamma \) than CD4^+ T cells, as detected using ELISPOT assays. This indicates that the CD4^+ T cells make more IFN-\( \gamma \) on a per cell basis than do the CD8^+ T cells. No IL-4, IL-5, or IL-10 was detected in any of these cultures, further showing that very little virus-specific Th2 cytokines are produced during an acute LCMV infection.

When splenocytes from LCMV-immune mice (day 180 p.i.) were used in the ELISPOT assay, the frequency of unsorted and sorted CD8^+ T cells secreting IFN-\( \gamma \) in
the 20 h assay was greatly reduced compared to cells isolated during the acute LCMV infection. Using this assay, I was unable to detect any CD4+ T cells secreting IFN-γ from the memory mice. This reduced frequency likely reflects a decrease in the activation status of these cells as compared to the highly activated T cells during the acute infection. Thus, the ELISPOT assay performed here using LCMV-infected PEC as APC probably does not provide a strong enough stimulus to induce memory CD4+ T cells to secrete IFN-γ. I will introduce other strategies later in this thesis that are capable of detecting IFN-γ production by memory CD4+ T cells following a stronger stimulus with peptides in the presence of IL-2, an observation that would also be consistent with the activation status of the memory cells discussed here.

F. Brief summary of Chapter III. I have shown in this chapter that a large proportion of the CD4+ T cells express activation markers during the acute LCMV infection as compared to naive mice. In addition, I provide data showing that 25% of the CD4+ T cells are blast-sized by day 7 p.i. and that there is an increased number of CD4+ T cells that have entered the cell cycle. I also provide evidence that CZ-1, a novel mAb produced in our laboratory (Vargas-Cortes et al., 1992) (Brutkiewicz et al., 1993), represents a new activation and memory marker for mouse CD4+ T cells. Taken together, these results suggest that a high percentage of the CD4+ T cells become activated following LCMV infection. In this chapter I have also provided evidence that LCMV
infection induces primarily a Th1 cytokine profile as determined by ELISA and ELISPOT assays. Moreover, I provide data that suggest that recent reports demonstrating IL-4-production following LCMV infection (Su et al., 1998; Whitmire et al., 1998) may be inaccurate and due to false positives caused by FCS antigens present in the inoculum.
CHATER IV

EXAMINATION OF THE LCMV-SPECIFIC CD4+ T CELL FREQUENCY FROM ACUTE INFECTION INTO MEMORY

The previous chapter demonstrated that CD4+ T cells become activated during an acute LCMV infection as judged by cell size, increased DNA content, expression of various activation markers, and secretion of cytokines. Although some of these assays indicated that as many as 25-50% of the CD4+ T cells in the spleen were activated, <1% of the CD4+ T cells scored as virus-specific cells in the ELISPOT assays measuring IFN-γ production. LDA have often been used in order to quantitate the frequency of virus-specific T cells. In the case of CD8+ CTL, these assays rely on the ability of CD8+ T cells to lyse virus-infected target cells in very sensitive 51Cr-release assays. However, CD4+ T cells during LCMV-infection are not normally cytotoxic (Muller et al., 1992; Oxenius et al., 1998). Recent work in the Sendai and influenza virus systems has led to the establishment of a CD4 LDA method that is based on IL-2 production by the virus-specific CD4+ T cells (Ewing et al., 1995; Topham et al., 1996; Tripp et al., 1997). This method, in contrast to the phenotypic studies presented in Chapter III, allows for a measurement of the magnitude of the virus-specific CD4+ T cell response. Therefore, I
adapted this method to measure LCMV-specific CD4+ Thp and present in this chapter a quantitation of the virus-specific CD4+ T cell response. In this chapter, I will show that the LCMV-specific memory CD4+ Thp frequency remains extremely stable into long-term immunity. In addition, I will provide data using newer, more sensitive assays based on intracellular cytokine production, that show that LCMV induces an even more profound CD4+ T cell response than has been previously realized.

A. Quantitation of the CD4+ Thp frequency during the acute LCMV infection into memory. Figure IV-1 shows a typical regression line obtained from C57BL/6 mice during the acute infection with LCMV (day 7 p.i.) and into memory (day 60 p.i.) using the IL-2-based LDA. Kinetic studies revealed that peak frequencies of CD4+ Thp during the acute LCMV infection and into memory were found after at least 48 hrs following the initiation of the culture (Fig. IV-1). The LDA results shown here suggest that memory T cells require at least 48 hrs of stimulation to score in these assays, providing an explanation as to why I saw a decline in the frequency of IFN-γ-secreting cells that could be detected using the ELISPOT assay in Chapter III.

Analysis of CD4+ Thp frequencies during the acute LCMV-infection and on into memory showed that the CD4+ Thp frequencies remain quite stable for at least 1 year p.i. (Table IV-1). The LCMV-specific CD4+ Thp frequency rose from <1/100,000 in a naive animal (day 0) to approximately 1/600 by day 10 p.i. with LCMV. This peak in the
Figure IV-1. Regression lines for purified CD4$^+$ T cells recovered from the spleens of C57BL/6 mice 7 and 60 days p.i. with LCMV. Limiting dilution assays were set up as described in the Materials and Methods, and culture supernatants were removed at the various times indicated and assessed for lymphokine production using the CTLL-2 indicator cell line.
### Table IV-1. LCMV-Specific Thp Frequencies During the Acute Infection and on into Memory

<table>
<thead>
<tr>
<th>Days P.I.</th>
<th>Exp. No.</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>Mean&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>1</td>
<td>337,756 (188,569-1,617,256)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>103,113 (67,082-222,763)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96,806 (73,680-141,085)</td>
<td>69,808 (51,469-108,455)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>202</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>468,333 (228,443-9,346,498)</td>
<td>301,747 (192,446-698,411)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>1</td>
<td>61,167 (27,528-275,580)</td>
<td>4,364 (3,272-6,556)</td>
<td>1,880</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>43,749 (27,871-101,666)</td>
<td>4,328 (3,199-6,690)</td>
<td>1,334</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11,206 (5,787-175,757)</td>
<td>2,966 (2,388-3,911)</td>
<td>1,471</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7,439 (5,352-12,192)</td>
<td>807 (654-1,053)</td>
<td>9,481</td>
<td></td>
</tr>
<tr>
<td>Day 9</td>
<td>1</td>
<td>43,185 (25,579-138,529)</td>
<td>976 (726-1,491)</td>
<td>13,276</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>27,815 (17,333-70,382)</td>
<td>665 (554-831)</td>
<td>8,618</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10,524 (7,699-16,622)</td>
<td>531 (432-689)</td>
<td>16,554</td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>1</td>
<td>19,445 (12,618-42,372)</td>
<td>858 (695-1,120)</td>
<td>14,698</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9,348 (6,771-15,097)</td>
<td>496 (400-654)</td>
<td>23,004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11,122 (6,977-27,404)</td>
<td>574 (439-697)</td>
<td>21,951</td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>17.4</td>
<td>15.4</td>
<td>41.3</td>
<td>18.0</td>
<td>54.8</td>
<td>24.8</td>
</tr>
<tr>
<td>154.1</td>
<td>41.3</td>
<td>19.4</td>
<td>18.0</td>
<td>14.0</td>
<td>14.0</td>
</tr>
<tr>
<td>5,423</td>
<td>6,749</td>
<td>1,681</td>
<td>1,435</td>
<td>1,350</td>
<td>1,381</td>
</tr>
<tr>
<td>1,179</td>
<td>1,455</td>
<td>7,05</td>
<td>1,350</td>
<td>1,381</td>
<td>5,315</td>
</tr>
</tbody>
</table>

Mean Thp for cultures stimulated with LCMV-infected APC.

Estimated total number of LCMV-specific Thp per spleen calculated from the frequencies and cell counts.

95% confidence limits.

Not significantly different (within the 95% confidence limits) from uninfected controls.

ND, not done.
CD4+ Thp frequency by day 10 p.i. corresponds with that of the activation markers CD44 and CD62L discussed in Chapter III. Interestingly, the LCMV-specific CD4+ Thp frequency per CD4+ T cell only dropped 2-fold from the peak of the acute response (days 9-11 p.i.) into long-term memory. This relatively small reduction in the CD4+ Thp frequency mirrors the 2-fold drop in frequency that our laboratory has previously reported for the CD8+ T cell response during LCMV-infection (Selin et al., 1996). In fact, on a per spleen basis, the decline in the total number of virus-specific memory CD4+ T cells is even less than the decline in the total number of virus-specific memory CD8+ T cells, because the total number of CD4+ T cells in the spleen remains the same between the peak of the acute infection and memory, whereas the total number of CD8+ T cells decreases significantly (see Fig. III-1).

The CD4+ Thp frequencies obtained against both uninfected and LCMV-infected APC (Table IV-1 and Table IV-2) are presented because there is consistently a low level response against uninfected APC. Several additional controls were performed to ensure that I was measuring LCMV-specific CD4+ Thp producing IL-2 in these assays. FACS-purified CD4+ T cells from a pool of 3 mice injected with a BHK21 cell supernatant yielded low frequencies to either uninfected (1/20,807) and LCMV-infected APC (1/22,439). Even though I used FACS-purified CD4+ T cells in these LDA, I could not rule out that a very small contamination (<1%) by CD8+ T cells might be present in the cultures and release IL-2 in response to the virus-infected APC. Using this IL-2-based
Table IV-2. Effect of Anti-CD4 or Anti-IL-4 mAb on LCMV-Specific Thp Frequencies

<table>
<thead>
<tr>
<th>Days</th>
<th>Experiment Number</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>Anti-CD4 mAb</th>
<th>Anti-IL-4 mAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.I.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>1</td>
<td>19,445 (12,618-42,372)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>858 (695-1,120)</td>
<td>20,638 (13,854-40,439)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9,348 (6,771-15,097)</td>
<td>496 (400-654)</td>
<td>21,234 (12,820-61,769)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Day 15</td>
<td>1</td>
<td>22,202 (14,264-50,069)</td>
<td>2,398 (1,899-3,254)</td>
<td>ND</td>
<td>2,416 (1,932-3,226)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>NR&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2,093 (1,663-2,822)</td>
<td>ND</td>
<td>1,710 (1,375-2,261)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 180</td>
<td>1</td>
<td>19,141 (12,322-42,852)</td>
<td>685 (501-768)</td>
<td>ND</td>
<td>1,523 (1,188-2,121)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24,368 (14,380-79,802)</td>
<td>2,887 (2,346-3,756)</td>
<td>ND</td>
<td>2,605 (2,076-3,499)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>95% confidence limits  
<sup>b</sup>Not significantly different (within the 95% confidence limits) from uninfected controls.  
<sup>c</sup>ND, not done.  
<sup>d</sup>Not significantly different (within the 95% confidence limits) from cultures with LCMV-infected APC.  
<sup>e</sup>NR, no response.
LDA method, FACS-purified CD8\(^+\) T cells at day 10 p.i. yielded frequencies of 1/2,650 and 1/2,388 in 2 independent experiments. Thus, CD8\(^+\) T cells are capable of making enough IL-2 to be detected in this assay. However, as shown in Table IV-2, addition of anti-CD4 mAb in my LDA using sorted CD4\(^+\) T cells, blocked all of the virus-specific IL-2 production, returning the CD4\(^+\) Thp frequency to a level comparable to that obtained against uninfected APC. Finally, because there was the possibility that the CTLL-2 cells used to monitor IL-2 production in these LDA could also be detecting IL-4, I added anti-IL-4 mAb to the assays. Table IV-2 shows that there was no effect (within the 95% confidence limits) in 3 of the 4 experiments when anti-IL-4 mAb was added to the LDA cultures. In one experiment, using LCMV-immune animals, the presence of the anti-IL-4 mAb only slightly (within the 95% confidence limits) reduced the resulting precursor frequency. In 2 additional experiments, I used the very IL-4-sensitive CT.4S cell line in the LDA. No IL-4 could be detected using this indicator cell line at day 15 or 240 p.i. in the LDA. The ELISPOT and ELISA data presented in Chapter III, along with the anti-IL-4 blocking experiments and use of the CT.4S cells discussed here, suggest that the LCMV-specific CD4\(^+\) T cells are primarily of a Th1 phenotype.

**B. Quantitation of the peptide-specific CD4\(^+\) Thp frequency from the peak of the acute infection into long-term memory.** Table IV-3 shows the peptide-specific CD4\(^+\) Thp frequencies for the two known LCMV encoded MHC class II-restricted
Table IV-3. LCMV-Specific Thp Frequencies to the LCMV MHC Class II-Restricted Peptides$^a$

<table>
<thead>
<tr>
<th>Day P.I.</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
<th>NP-Pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 10</td>
<td>&gt;120,000</td>
<td>1,536 (1,191-2,160)$^b$</td>
<td>931 (740-1,256)</td>
<td>5,460 (3,945-7,242)</td>
</tr>
<tr>
<td>Day 450</td>
<td>47,551 (34,919-74,502)</td>
<td>1,505 (1,172-2,100)</td>
<td>2,055 (1,666-2,680)</td>
<td>10,762 (3,945-7,242)</td>
</tr>
<tr>
<td>Day 10</td>
<td>&gt;240,000</td>
<td>5,402 (4,199-7,572)</td>
<td>1,423 (1,112-1,977)</td>
<td>3,785 (3,148-4,747)</td>
</tr>
<tr>
<td>Day 450</td>
<td>60,986 (33,657-324,385)</td>
<td>6,125 (4,665-8,912)</td>
<td>5,118 (4,137-6,707)</td>
<td>12,264 (9,265-18,134)</td>
</tr>
<tr>
<td>Day 10</td>
<td>22,243 (16,222-35,370)</td>
<td>1,073 (854-1,444)</td>
<td>301 (230-434)</td>
<td>2,028 (1,590-2,799)</td>
</tr>
<tr>
<td>Day 540</td>
<td>&gt;240,000</td>
<td>1,288 (1,046-1,674)</td>
<td>2,108 (1,641-2,941)</td>
<td>6,859 (5,444-9,263)</td>
</tr>
</tbody>
</table>

$^a$CD4 LDA were performed as described in the *Materials and Methods*. GP- and NP-pulsed APC refer to PEC that have been pulsed with 5 μg/ml of either the GP61-80 or NP309-328 peptide.

$^b$95% confidence limits.
peptides during the peak of the acute CD4+ T cell response and into long-term memory. The stability of the virus-specific CD4+ Thp frequencies up to 18 months p.i. following stimulation with LCMV-infected PEC shown here extends on the data presented in Table IV-1 regarding the stability of CD4+ Thp frequencies into long-term memory. The CD4+ Thp frequency to each of the two LCMV MHC class II-restricted peptides dropped only 2- to 7-fold from the peak of the CD4+ T cell response into memory (Table IV-3). It is unclear why the peptide-specific CD4+ Thp frequency yields such a variable drop-off from the peak of the response into memory. Using the peptides as the stimulus may allow me to detect lower affinity virus-specific CD4+ T cells than I would be able to detect using whole virus. It is clear that a higher frequency of CD4+ T cells score as virus-specific in the LDA using the two peptides as compared to using whole virus. However, the peptide-specific CD4+ Thp frequencies shown here do not decline between days 450 and 540 p.i., agreeing with the results discussed above using whole virus showing that the LCMV-specific CD4+ Thp frequency remains stable into long-term immunity.

C. Phenotype of LCMV-specific CD4+ Thp. The above experiments demonstrate that, like the LCMV-specific CD8+ CTLp frequency, the LCMV-specific CD4+ Thp frequency is extremely stable for essentially the life of the mouse. Previous work from our laboratory has shown that the LCMV-specific memory CD8+ CTLp express
predominantly a CD44hi, CD62Lhi, CD11bhi phenotype (Razvi et al., 1995). However, some memory CD8+ CTLp may be found in the T cells expressing the reciprocal levels of these markers. In addition, some of the CD44hiCD62LhiCD11bhi LCMV-specific memory CD8+ CTLp were blast-sized cells expressing IL-2 receptors (Razvi et al., 1995). This suggests that at least a portion of the memory T cells are cycling. In order to analyze the LCMV-specific memory CD4+ Tp phenotype, CD4+ T cells obtained from LCMV-immune mice were sorted for CD4+ T cells expressing one of a panel of activation/memory markers (Table IV-4). The virus-specific memory CD4+ Tp expressed predominantly a CD44hiCD45RBloCD49dhiCD62LhiCZ-1hi phenotype. The least discriminating antigen in these experiments seemed to be CD45RB, often used as a marker for memory CD4+ T cells (Gray, 1993). A fairly high frequency of LCMV-specific memory CD4+ Tp were found in both the CD62Lhi and CD62Llo subsets. LCMV-virus-specific memory CD8+ CTLp have been shown to regain expression of CD62L over time (Razvi et al., 1995), and it seems that LCMV-specific memory CD4+ Tp also regain expression of this marker. This is important as CD62L regulates T cell migration by mediating lymphocyte attachment to the high endothelial venules of peripheral lymph nodes (Gallatin et al., 1983). The two best markers at identifying LCMV-specific CD4+ Tp were CD49d and CZ-1. Expression of CD49d on a high frequency of Sendai virus-specific memory CD4+ Tp has recently been reported (Ewing et al., 1995). The results obtained here with CZ-1 expand on my earlier characterization
### Table IV.4: Phenotype of LCMV-Infected CD4+ T Cell Precursors

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-Infected APC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4+CD62L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4+CD62L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4+CD44</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD4+CD44</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LCMV Imm</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reciprocal of Thp Frequency</th>
<th>1.498 (1.162-2.788)</th>
<th>5.315 (2.860-3.716)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.916 (4.839-7.617)</td>
<td>1.4338 (1.1761-18.362)</td>
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<tr>
<td>11.782 (7.947-1.4839)</td>
<td>13.504 (10.914-1.7704)</td>
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<tr>
<td>3.390 (2.727-4.476)</td>
<td>34.437 (18.340-28.1475)</td>
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<table>
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<tr>
<th>Reciprocal of Thp Frequency</th>
<th>651 (5.46-8.07)</th>
<th>10.620 (6.915-22.879)</th>
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<tr>
<td>2.422 (1.839-2.749)</td>
<td>11.189 (8.734-15.663)</td>
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<tr>
<td>63.3 (5.41-7.64)</td>
<td>9.709 (6.329-20.831)</td>
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<tr>
<td>1.650 (1.395-2.135)</td>
<td>7.905 (5.699-12.897)</td>
<td></td>
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</tbody>
</table>

**LCMV Imm**
| CD4⁺CD45RB⁺ | 36,042 (23,139-81,475) | 3,267 (2,450-4,903) |
| CD4⁺CD45RB⁻ | NR⁴ | 1,542 (1,222-2,089) |

| CD4⁺CD45RB⁺ | 63,620 (46,075-102,744) | 2,982 (2,303-4,227) |
| CD4⁺CD45RB⁻ | 5,341 (4,123-7,579) | 7,094 (5,339-10,568)⁵ |

| CD4⁺CZ-1⁺ | 30,085 (20,012-60,573) | 467 (392-577) |
| CD4⁺CZ-1⁻ | 11,668 (9,812-14,389) | 2,350 (1,805-3,369) |

| CD4⁺CD45RB⁺ | 45,168 (32,309-75,034) | 8,621 (6,514-12,739) |
| CD4⁺CD45RB⁻ | NR | 38,020 (21,980-140,663) |

| CD4⁺CZ-1⁺ | NR | 4,690 (3,766-6,216) |
| CD4⁺CZ-1⁻ | NR | 55,873 (35,602-129,746) |

| CD4⁺CD49d⁺ | 7,414 (5,803-10,268) | 349 (277-473) |
| CD4⁺CD49d⁻ | 22,306 (18,843-27,331) | 2,442 (1,964-3,228) |
Splenocytes from LCMV-immune mice (6-8 months) were stained and sorted into two populations based on CD4 and either CD44, CD62L, CD45RB, CD49d, or CZ-1 expression as described in the Materials and Methods. CD4 LDA were then performed using these two FACS-purified populations.

95% confidence limits.

Not significantly different (within the 95% confidence limits) from uninfected controls.

NR, no response.
of this novel marker by confirming that it is indeed preferentially expressed on the LCMV-specific memory CD4+ Thp.

**D. Intracellular staining of cytokine-producing CD4+ T cells.** As mentioned in the introduction, two new techniques have been developed to measure antigen-specific T cell populations. Recent studies using MHC class I tetramers loaded with immunodominant viral peptides and using intracellular staining for IFN-γ following virus-peptide stimulation have revealed that a significantly higher percentage of CD8+ T cells can be scored as virus-specific than has previously been detected using LDA (Butz and Bevan, 1998; Flynn et al., 1998; Lalvani et al., 1997; Murali-Krishna et al., 1998). Although the total CD4+ T cell number remains relatively stable during the acute LCMV infection (see Fig. III-1), there is an increase in the percentage of blast-sized CD4+ T cells, of CD4+ T cells with increased DNA content, and of CD4+ T cells expressing various activation and adhesion molecules, as shown in Chapter III (Varga and Welsh, 1998). Using ELISpot assays (in the absence of added IL-2) to assess IFN-γ production at the single-cell level, I have shown that the frequency of sorted CD4+ T cells that produced IFN-γ when stimulated with LCMV-infected PEC (see Table III-3), was similar to the CD4+ Thp frequency in LDA measuring IL-2-producing cells shown in Tables IV-1 and IV-3. Thus, <1% of the CD4+ T cells can be identified by LDA or ELISPOT analysis as LCMV-specific.
Figure IV-2. Intracellular IFN-γ and IL-4 expression of virus-induced CD4⁺ T cells following stimulation with PMA and ionomycin. Splenocytes from the indicated days p.i. were stimulated with PMA and ionomycin for 2 hr before the addition of brefeldin A for an additional 2 hr as described in the Materials and Methods. The cells were gated on CD4⁺ cells, and the values shown represent the percentage of CD4⁺ cells that expressed a given cytokine. Means ± standard deviations for time points from 2 separate experiments with 2 individual mice per experiment are shown (n=4 per group).
Figure IV-2 shows that a much higher frequency (>20%) of CD4\(^+\) T cells from mice acutely infected with LCMV make IFN-\(\gamma\) when nonspecifically stimulated with PMA and ionomycin. There was no significant increase in the percentage of IL-4-producing CD4\(^+\) T cells during the acute LCMV infection, in agreement with my earlier results presented in Chapter III using ELISA and ELISPOT assays. Thus, there is a much larger percentage of CD4\(^+\) T cells that are expressing an activated cell phenotype, that are blast-sized and have entered the cell cycle, and that produce IFN-\(\gamma\) following polyclonal stimulation, than can be accounted for by using either the LDA or ELISPOT assay (Figs. III-2, III-3, III-14, Tables III-3, and IV-1).

E. Intracellular staining of peptide-specific IFN-\(\gamma\)-producing CD4\(^+\) T cells. The above experiments show that at least 20-25% of the CD4\(^+\) T cells in the acute LCMV infection produce IFN-\(\gamma\) when nonspecifically stimulated with PMA and ionomycin. This assay detects cells that have been previously activated in vivo, as the short 4 hr stimulation with PMA and ionomycin is not long enough to activate a naive T cell or a resting memory T cell to produce IFN-\(\gamma\), as LCMV-immune mice do not exhibit increased frequencies of IFN-\(\gamma\)-producing CD4\(^+\) T cells above naive control mice (data not shown). Moreover, this assay relies on polyclonal stimulation of the activated T cells and therefore does not address the specificity of the responding cells. As mentioned earlier, one of the new techniques to measure antigen-specific T cells is by using the intracellular
IFN-γ assay following peptide stimulation (Murali-Krishna et al., 1998). I adapted this method to measure LCMV peptide-specific CD4+ T cells by using the two known LCMV MHC class II-restricted CD4 peptides. This assay is performed by stimulating the T cells in vitro for 5 hr in the presence of peptide, IL-2, and brefeldin A (to allow the cytokines to accumulate intracellularly). The peptide-specific intracellular IFN-γ assay, displayed in Figure IV-3, shows that >10% of the CD4+ T cells at day 9 p.i. were specific for either of the two LCMV MHC class II-restricted peptides. CD4+ T cells produced no IFN-γ in the absence of peptide, and peptide-stimulated CD4+ T cells did not demonstrate any significant intracellular staining above background with an isotype control mAb. Figure IV-4 shows that virtually all of the GP61-80-specific CD4+ T cells that stained positive for IFN-γ were also CD44hi, consistent with an activated cell phenotype (Bradley et al., 1993). Figure IV-5 shows a time course in which the IFN-γ-producing cells peak at day 9 p.i., but are still detectable into long-term memory.

I performed several experiments using mAb specific to Th2 cytokines such as IL-4, IL-5, and IL-10 and failed to detect an increase, above the levels obtained with the appropriate isotype-matched control mAb, in the intracellular expression of any of these cytokines following peptide stimulation (data not shown). To further make sure that no significant virus-specific IL-4 was being produced following LCMV infection, I examined the expression of intracellular IFN-γ and IL-4 following LCMV infection of C57BL/6 mice genetically deficient in CD8+ T cells. Figure IV-6 shows that there is a significant
Figure IV-3. Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells. Splenocytes from LCMV-infected (day 9) C57BL/6 mice were stimulated with or without one of the two LCMV MHC class II-restricted peptides in the presence of IL-2 and brefeldin A for 5 hr as described in the Materials and Methods. The numbers shown indicate the percentage of CD4+ T cells that stained positive for intracellular IFN-γ as represented by the R1 gate. Data are representative of four experiments.
Figure IV-4. CD44 expression on peptide-specific CD4+ T cells staining positive for intracellular IFN-γ. Splenocytes from LCMV-infected (day 9) mice were cultured in vitro with GP61-80 in the presence of IL-2 and brefeldin A for 5 hr as described in the Materials and Methods. In the top panel the numbers shown in the R1 gate indicate the percentage of splenocytes that are positive for the A isotype control mAb, B intracellular IFN-γ stain, or C control IFN-γ staining of cells from day 9-infected mice pre-stained with unlabeled anti-IFN-γ mAb. The histograms in the bottom panel (D, E) represent the cell surface expression of CD44 on the two gated populations from B. Data are representative of 4 separate experiments.
Figure IV-5. Intracellular IFN-γ expression of LCMV peptide-specific CD4⁺ T cells during an acute LCMV infection into memory. Splenocytes from the indicated days p.i. were stimulated with one of the two LCMV MHC class II-restricted peptides in the presence of IL-2 and brefeldin A for 5 hr, as described in the Materials and Methods. Background staining with the appropriate isotype-matched control mAb was subtracted from each individual. Means ± standard deviations for time points from 4 separate experiments with 2 individual mice per experiment are shown (n=8 per group).
Figure IV-6. Intracellular IFN-γ and IL-4 expression of LCMV peptide-specific CD4+ T cells from CD8 knock-out mice. Splenocytes from LCMV-infected (day 9) C57BL/6-CD8KO mice were stimulated with one of the two LCMV MHC class II-restricted peptides in the presence of IL-2 and brefeldin A for 5 hr as described in the Materials and Methods. The numbers shown indicate the percentage of CD4+ T cells that stained positive for intracellular IFN-γ or IL-4 as represented by the R1 gate. Data are representative of 2 experiments.
frequency of IFN-\(\gamma\)-producing cells at day 9 p.i. with no increase in the frequency of IL-4-producing cells. Taken together, these results are in general agreement with the LDA and ELISPOT assays presented earlier showing an increase in the frequency of virus-specific cells secreting Th1 cytokines with no increase in the frequency of virus-specific cells secreting Th2 cytokines. Thus, the main difference between the LDA or ELISPOT results and the intracellular cytokine stains is that the latter assay scores a much higher frequency of CD4\(^+\) T cells as being virus-specific.

The time course presented in Figure IV-5 using the intracellular IFN-\(\gamma\) assay looks similar in kinetics to that described earlier using the ELISPOT assay (see Fig. III-14). In contrast to using LCMV-infected PEC as APC in the ELISPOT assay, the intracellular IFN-\(\gamma\) assay uses peptide in the presence of IL-2 to activate the T cells, and this likely explains why the intracellular IFN-\(\gamma\) assay detects a much higher frequency of virus-specific CD4\(^+\) T cells as compared to the ELISPOT assay. This explanation is supported by recent studies showing that the intracellular cytokine and ELISPOT assays yield similar results when peptides and IL-2 are used as the T cell stimulus in the ELISPOT assay (Murali-Krishna et al., 1998). The same reasoning may explain the disparity between the frequency of virus-specific CD4\(^+\) T cells detected using the LDA as compared to the intracellular IFN-\(\gamma\) assay. I fail to detect IL-2-secreting cells in the intracellular cytokine assays discussed above when brefeldin A is used to allow the cytokines to accumulate intracellularly. A recent report in the LCMV system has also
failed to detect them using similar assays following anti-CD3 stimulation (Su et al., 1998). Monensin is another compound that may be used in the intracellular cytokine assays to disrupt the Golgi and allow cytokines to accumulate. Monensin has been reported to enhance the detection of Th2 cytokines such as IL-4 and IL-10 as compared to brefeldin A. However, I failed to detect an increase, above the levels obtained with the appropriate isotype-matched control mAb, in the intracellular expression of Th2 cytokines such as IL-4, IL-5, or IL-10 during an acute LCMV infection following peptide stimulation in the presence of monensin (data not shown). Interestingly, the use of monensin did allow for the detection of IL-2-producing cells in the intracellular cytokine assay that were not previously observed when using brefeldin A. Table IV-5 shows that approximately 2% of the CD4+ T cells produce IL-2 when stimulated with either of the two LCMV MHC class II-restricted peptides. Treatment with monensin does result in a lower frequency of cells that secrete IFN-γ (approximately 5% vs. 10% with brefeldin A).

The frequency of CD4+ T cells capable of making TNF-α was similar to that of IFN-γ (both approximately 5%) following stimulation with the LCMV MHC class II-restricted peptides in the presence of IL-2 and monensin. This assay allows a comparison of the frequency of virus-specific IL-2-secreting CD4+ T cells obtained with the LDA. The intracellular stain using monensin is able to detect a higher frequency of virus-specific IL-2-producing cells as compared to the LDA discussed above. This discrepancy may be due to the addition of exogenous IL-2 in the intracellular stain which may, in combination
<table>
<thead>
<tr>
<th>Day P.I.</th>
<th>Frequency of CD4+ IL-2+ Cells</th>
<th>Frequency of CD4+ IFN-γ Cells</th>
<th>Frequency of CD4+ TNF-α Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>&lt;0.20</td>
<td>0.33±0.20</td>
<td>0.31±0.21</td>
</tr>
<tr>
<td>Day 7</td>
<td>2.15±0.43</td>
<td>0.42±0.20</td>
<td>0.47±0.35</td>
</tr>
<tr>
<td>Day 9</td>
<td>1.70±0.74</td>
<td>0.50±0.26</td>
<td>0.73±0.52</td>
</tr>
<tr>
<td>Day 11</td>
<td>1.31±0.96</td>
<td>0.60±0.45</td>
<td>0.51±0.37</td>
</tr>
<tr>
<td>Day 15</td>
<td>0.57±0.47</td>
<td>0.38±0.26</td>
<td>0.37±0.47</td>
</tr>
<tr>
<td>Day 180</td>
<td>0.33±0.13</td>
<td>0.35±0.16</td>
<td>0.37±0.13</td>
</tr>
</tbody>
</table>

Intracellular cytokine assays were performed on splenocytes from the indicated days p.i. with LCMV that were stimulated with one of the two LCMV MHC class II-restricted peptides in the presence of IL-2 and monensin for 5 h, as described in the Materials and Methods. Background staining with the appropriate isotype-matched control mAb was subtracted from each individual. Means± standard deviations for time points from 3 separate experiments with 2 individual mice per experiment are shown (n=6 per group).
with using the MHC class II-restricted peptides, allow for the detection of lower affinity virus-specific CD4⁺ T cells that are not detectable using the LDA. It is difficult to directly test this as the LDA is measuring IL-2 production by the virus-specific CD4⁺ T cells using an IL-2-sensitive indicator cell line and thus prevents the addition of exogenous IL-2 in these assays. The intracellular stain avoids this problem as human recombinant IL-2 is used to stimulate the virus-specific T cells whereas the IL-2-producing cells are detected using a mAb specific for murine IL-2.

Using the intracellular IFN-γ assay, the percentage of CD4⁺ T cells that are LCMV peptide-specific drops 5- to 7-fold from the peak of the response into long-term immunity. This agrees with the results I obtained in the LDA using these two peptides but contrasts with the consistent 2-fold drop I observe in the LDA using whole virus. This difference may be due to the detection of lower affinity T cells following peptide stimulation as compared to using whole virus. However, like the results obtained using the IL-2-based LDA, the intracellular IFN-γ assay revealed that the LCMV peptide-specific CD4⁺ T cell frequency remains stable into long-term immunity. The intracellular IFN-γ stain following stimulation with either of the two known MHC class II-restricted peptides shown here scores >10% of the CD4⁺ T cells as LCMV-specific. In contrast, Figure IV-2 demonstrated that 20-25% of the CD4⁺ T cells could make IFN-γ following polyclonal stimulation. The total number of virus-specific CD4⁺ T cells may be higher than I show here, since only two CD4 peptides were used. It is likely that the CD4⁺ T
cell response to LCMV includes other MHC class II-restricted epitopes which have yet to be identified. Thus, these new sensitive assays reveal a previously unappreciated profound antigen-specific CD4⁺ T cell response during viral infections.

**F. Brief summary to Chapter IV.** In this Chapter I make two novel observations. First, I provide data showing that the LCMV-specific memory CD4⁺ Thp frequency remains extremely stable into long-term immunity. Second, I provide evidence using newer, more sensitive assays based on intracellular cytokine production, that LCMV induces an even more profound CD4⁺ T cell response than has been previously realized. It is possible that this second observation may be an unappreciated feature of viruses in general, as LCMV was a virus not previously thought to be a strong inducer of CD4⁺ T cells.
CHAPTER V

STABILITY OF THE LCMV-SPECIFIC CD4+ T CELL FREQUENCY FOLLOWING HETEROLOGOUS VIRUS INFECTIONS¹

Recent work from our laboratory has shown that serologically unrelated viruses (referred to as heterologous viruses) can elicit cross-reactive CD8+ CTL responses that participate in the early stages of the immune response when mice immune to one virus are challenged with another virus (Selin et al., 1994; Selin and Welsh, 1994). Mice immune to LCMV synthesize approximately 10-fold less PV or VV upon challenge, when compared to naive mice, and this protection can be adoptively transferred into naive mice by T cells obtained from LCMV-immune mice (Selin et al., 1998; Welsh et al., 1996). Previous studies have shown that antigen-stimulated memory T cells can secrete much higher quantities of cytokines than recently stimulated naive T cells undergoing a primary immune response (Bradley et al., 1993). Therefore, in order to further define the factors leading to this increased immunity to heterologous viruses, I utilized ELISA assays and flow cytometric analyses of intracellular IFN-γ to examine cytokine production at the single-cell level following infection of immune mice with heterologous viruses. I will

¹ Some of the work performed in this chapter was done in collaboration with Dr. L.K. Selin (Selin et al., 1998).
show that there is an increase in the frequency of IFN-\(\gamma\)-secreting cells and IFN-\(\gamma\) protein in the peritoneal cavity of LCMV-immune mice challenged with VV as compared to control-immune mice infected with VV.

More recent results from our laboratory have shown that heterologous virus infections may also disrupt the long-term homeostasis of the CD8\(^+\) T cell memory pool by reducing the frequency of CD8\(^+\) CTLp to earlier viruses (Selin et al., 1996). The data presented in Chapter IV demonstrated that the virus-specific CD4\(^+\) Thp frequency is extremely stable into long-term immunity following a single infection with LCMV. In this Chapter, I questioned whether heterologous virus infections would perturb the stable LCMV-specific CD4\(^+\) Thp memory pool. I will show that the CD4\(^+\) Thp frequency is resistant to reduction by heterologous virus infections, even under conditions that reduce the LCMV-specific CD8\(^+\) CTLp frequency.

A. T cell distribution and activation phenotype of CD4\(^+\) T cells following different virus infections (LCMV, PV, VV, MCMV). Figure V-1 shows the percentage of CD4\(^+\) and CD8\(^+\) T cells in the spleen of C57BL/6 mice acutely infected with either LCMV, VV, PV, or MCMV. Also shown is the increase in the total leukocyte number in the spleen that occurs during each of these virus infections. As shown in Chapter III (see Fig. III-1), LCMV infection results in a 2-fold increase in the total leukocyte number in the spleen and a conversion of the CD4 to CD8 ratio from 2:1 to 1:2-3. Each of the other
Figure V-1. CD4/CD8 ratio during acute LCMV, VV, PV, or MCMV infection. Cell numbers, expressed as cell counts per spleen (●), and cell phenotypes for the cells recovered from the spleens of mice infected with either LCMV, VV, PV, or MCMV. The cell phenotypes, expressed as percentages of total splenic leukocytes, were determined by FACS analysis, as described in the Materials and Methods. Means ± standard deviations for time points from 3 separate experiments with 2 individual mice per experiment are shown (n=6 per group).
viruses induce an increase in the total leukocyte number in the spleen as well as a conversion of the CD4 to CD8 ratio. However, none of the other viruses induce these changes to the extent LCMV infection does. I presented data in Chapter III showing that there is an increase in the proportion of CD4+ T cells expressing activation markers such as CD44 (see Fig. III-2 and Table III-1), consistent with an activated phenotype. Here I examined the extent of CD4+ T cell activation following an acute infection with either LCMV, VV, PV, or MCMV by determining the percentage of CD4+CD44hi cells in the spleen at various days p.i. The mean percentage of CD4+CD44hi cells from 6 individual mice per group were as follows: day 0: 22±4; LCMV day 7: 18±6; LCMV day 9:32±7; VV day 7: 30±5; VV day 9: 27±4; PV day 7: 20±5; PV day 9: 24±5; MCMV day 7: 37±11; MCMV day 9: 38±11. Thus, each of these viruses induce CD4+ T cells expressing an activated phenotype.

B. Increased frequency of IFN-γ protein and IFN-γ-secreting cells following a heterologous virus infection. Our laboratory has previously shown that memory CD8+ T cells from a previous viral infection can be stimulated by heterologous viruses (Selin et al., 1994; Selin and Welsh, 1994; Welsh et al., 1996), and I questioned here how challenging immune mice with heterologous viruses would influence cytokine production. LCMV-immune mice were challenged with VV, and the amount of IFN-γ in the peritoneal fluid was measured by ELISA at day 3 p.i. with VV, a period in which there should be
little contribution from naive T cells responding to the acute virus infection. As can be seen in Figure V-2, infection of LCMV-immune mice with VV (16,115±14,766 pg/ml) resulted in a >1,074-fold increase in IFN-γ protein levels in the peritoneal fluid at day 3 p.i. as compared to naive mice (<15 pg/ml) or LCMV-immune mice (<15 pg/ml), or a 5.4-fold increase in comparison to control-immune mice acutely infected with VV (2,979±3,600 pg/ml). LCMV-immune mice challenged with PV or VV-immune mice challenged with LCMV did not exhibit this enhanced production of IFN-γ (<15 pg/ml).

Further examination of the peritoneal cavity using FACS analysis revealed a significant recruitment of CD8⁺ (8-fold) and CD4⁺ (2.4-fold) T cells into this compartment in LCMV-immune versus control-immune mice 3 days p.i. with VV (Fig. V-3). In addition, most of the recruited cells were CD44hi, consistent with an activated and/or memory phenotype. I also examined the peritoneal cells for the expression of intracellular IFN-γ following stimulation with PMA and ionomycin. Figure V-3 shows that LCMV-immune mice challenged with VV exhibit a significant (p<0.01) increase in the number of IFN-γ-producing CD8⁺ (10-fold) and CD4⁺ (2.7-fold) T cells at 3 days p.i. as compared to control-immune mice challenged with VV. Experiments performed by Dr. L.K. Selin have shown that LCMV-immune mice have 10- to 300-fold lower VV titers in the spleen, fat pad, and liver 3-5 days p.i. with VV (Selin et al., 1998). T cell depletion experiments demonstrated that both CD4⁺ and CD8⁺ T cells were required to mediate this reduction in virus titers. Moreover, it was shown that LCMV-immune mice genetically deficient in
Figure V.2. Increased IFN-$\gamma$ protein levels in peritoneal lavage fluid of LCMV-immune mice versus control-immune mice 3 days after challenge with VV. Peritoneal lavages were performed and IFN-$\gamma$ protein was quantitated using an Endogen mouse IFN-$\gamma$ ELISA kit as per the manufacturer's instructions. IFN-$\gamma$ protein levels from 3 separate experiments with 4 individual mice per experiment are shown ($n=12$ per group). There was a statistically significant difference between the amount of IFN-$\gamma$ protein detected in the peritoneum of LCMV-immune as compared to control-immune mice 3 days after VV infection ($p<0.01$).
Increased numbers of IFN-γ-secreting CD8+ and CD4+ T cells in the peritoneum of LCMV-immune mice challenged with VV (day 3). Peritoneal cells were harvested from age-matched naive and LCMV-immune C57BL/6 mice challenged with VV (day 3) or from LCMV-immune mice challenged with a control supernatant (day 3). The total number of CD8+ and CD4+ T cells (white bars) and the total number of IFN-γ-secreting CD8+ and CD4+ T cells (black bars) were determined as described in the Materials and Methods. Means ± standard error of the mean for time points from 3 separate experiments with 4 individual mice per experiment are shown (n=12 per group). * indicates a statistically significant difference from the LCMV-immune+VV group (p<0.01). These experiments were performed in collaboration with Dr. L.K. Selin.
IFN-γ receptors failed to protect against VV infection but were still able to protect against PV infection (Selin et al., 1998). Thus, these experiments demonstrate that both CD8+ and CD4+ memory T cells are recruited to produce IFN-γ in LCMV-immune mice challenged with VV, and are able to offer enhanced protection by reducing VV titers early after infection.

C. LCMV-specific CD4+ Thp frequency does not decline following multiple heterologous virus infections. The above experiments suggest that CD4+ T cells become activated during each of these virus infections and in some cases, the memory CD4+ T cells may play a role in mediating increased resistance to a heterologous virus infection. Recent work from our laboratory has shown that heterologous virus infections can have a profound impact on CD8+ T cell memory pool by causing reductions in the CD8+ CTLp frequency to earlier virus infections (Selin et al., 1996). Here I wanted to ask the question of whether or not heterologous virus infections caused a similar perturbation in the memory CD4+ T cell compartment. Having already established a CD4 LDA method for measuring LCMV-specific CD4+ Thp and shown that the LCMV-specific CD4+ Thp frequency remains quite stable into long-term immunity (see Table IV-1), I examined the LCMV-specific CD4+ Thp frequency after 1-3 heterologous virus infections. Figure V-4 shows that the LCMV-specific CD4+ Thp does not decline following multiple heterologous virus infections (PV, VV, and MCMV). In addition, in
Figure V-4. Reduction of CD8⁺ CTLp but not CD4⁺ Thp following challenge of LCMV-immune mice with several heterologous virus infections. CD4 LDA were performed as described in the Materials and Methods on pooled spleen cells (3-4 mice) from mice immune to each of the viruses listed. The results shown represent 3 individual experiments in which each group of mice was sampled within the same LDA. In one experiment, the CD8⁺ CTLp frequency was determined from the same mice by Dr. L.K. Selin.
one of these assays, a CD8 LDA was performed by Dr. L.K. Selin in order to show that within these mice, the LCMV-specific CD8⁺ CTLp frequency does decline as she has previously reported (CD8⁺ CTLp declined 6-fold in LCMV+PV-immune mice and 18-fold in LCMV+PV+VV+MCMV-immune mice as compared to LCMV-immune mice). Figure V-5 shows similar results quantifying LCMV-specific CD4⁺ Thp and CD8⁺ CTLp in mice previously immunized with PV (PV+LCMV-immune mice) as compared to PV+LCMV+VV+MCMV-immune mice (CD8⁺ CTLp declined 2-fold in PV+LCMV+VV+MCMV-immune mice as compared to PV+LCMV-immune mice). I performed additional experiments using the LCMV MHC class II-restricted CD4 peptides in order to follow the fate of the peptide-specific CD4⁺ Thp under these same conditions. Table V-1 shows 3 independent experiments in which there is no significant decline in the LCMV-specific CD4⁺ Thp frequency to whole virus or to either of the two LCMV MHC class II-restricted CD4 peptides.

In order to examine if the order of the virus infections played a role, I performed similar experiments using two other virus sequences. Table V-2 shows, using PV+LCMV+VV+MCMV-immune mice, a similar lack of CD4⁺ Thp reduction to that discussed above for LCMV+VV+PV+MCMV-immune mice. Interestingly, Table V-3 shows, using VV+LCMV+PV+MCMV-immune mice that there is a slight drop (2-fold) in the LCMV-specific CD4⁺ Thp frequency to each of the LCMV MHC class II-restricted CD4 peptides in the first experiment and to the whole virus and to the peptides
Figure V-5. Reduction of CD8⁺ CTLp but not CD4⁺ Thp following challenge of PV+LCMV-immune mice with several heterologous virus infections. CD4 LDA were performed as described in the Materials and Methods on pooled spleen cells (3-4 mice) from mice immune to each of the viruses listed. The results shown represent 3 individual experiments in which each group of mice was sampled within the same LDA. In one experiment, the CD8⁺ CTLp frequency was determined from the same mice by Dr. L.K. Selin.
### Table V-1. LCMV-Immune Mice Challenged with Heterologous Viruses

<table>
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<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
<th>NP-Pulsed APC</th>
</tr>
</thead>
<tbody>
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<td>LCMV</td>
<td>6,067 (5,098-7,490)(^a)</td>
<td>1,751 (1,356-2,471)</td>
<td>1,842 (1,370-2,809)</td>
<td>5,106 (3,896-7,409)(^b)</td>
</tr>
<tr>
<td>L+P+V+M(^c)</td>
<td>6,118 (4,818-8,380)</td>
<td>1,736 (1,306-2,585)</td>
<td>1,275 (973-1,849)</td>
<td>4,260 (3,318-5,948)(^b)</td>
</tr>
<tr>
<td>LCMV</td>
<td>53,206 (29,878-242,693)</td>
<td>542 (396-862)</td>
<td>1,726 (1,351-2,390)</td>
<td>5,107 (3,945-7,242)</td>
</tr>
<tr>
<td>L+P+V+M(^c)</td>
<td>1,203 (1,050-1,411)</td>
<td>818 (589-1,336)(^b)</td>
<td>1,671 (1,231-2,602)(^b)</td>
<td>1,209 (845-2,126)(^b)</td>
</tr>
<tr>
<td>LCMV</td>
<td>9,475 (7,485-12,904)</td>
<td>816 (678-1,024)</td>
<td>894 (736-1,136)</td>
<td>3,445 (2,843-4,370)</td>
</tr>
<tr>
<td>L+P+V+M(^c)</td>
<td>11,135 (8,242-17,153)</td>
<td>1,342 (1,102-1,714)</td>
<td>970 (807-1,214)</td>
<td>3,991 (3,143-5,463)</td>
</tr>
</tbody>
</table>

\(^a\)95% confidence limits.
\(^b\)Not significantly different (within the 95% confidence limits) from uninfected controls.
\(^c\)Mice were infected with LCMV, PV, VV, and MCMV at two month intervals and sampled at least 2 months after the last infection.
Table V-2. PV-Immune Mice Challenged with Heterologous Viruses

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
<th>NP-Pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV+LCMV</td>
<td>6,944 (5,822-8,600)(^{a})</td>
<td>273 (220-358)</td>
<td>135 (99-210)</td>
<td>903 (715-1,224)</td>
</tr>
<tr>
<td>P+L+V+M(^{b})</td>
<td>2,111 (1,830-2,496)</td>
<td>625 (464-954)</td>
<td>1,348 (1,030-1,948)(^{c})</td>
<td>1,348 (1,087-1,772)</td>
</tr>
<tr>
<td>PV+LCMV</td>
<td>1,229 (1,025-1,536)</td>
<td>202 (163-265)</td>
<td>201 (162-262)</td>
<td>522 (423-678)</td>
</tr>
<tr>
<td>P+L+V+M(^{b})</td>
<td>543 (427-749)</td>
<td>116 (91-158)</td>
<td>208 (163-286)</td>
<td>317 (244-451)(^{c})</td>
</tr>
<tr>
<td>PV+LCMV</td>
<td>1,803 (1,470-2,332)</td>
<td>385 (299-537)</td>
<td>364 (298-467)</td>
<td>820 (683-1,025)</td>
</tr>
<tr>
<td>P+L+V+M(^{b})</td>
<td>450 (354-619)</td>
<td>261 (211-343)</td>
<td>232 (190-296)</td>
<td>426 (350-543)(^{c})</td>
</tr>
</tbody>
</table>

\(^{a}\)95% confidence limits.
\(^{b}\)Mice were infected with PV, LCMV, VV, and MCMV at two month intervals and sampled at least 2 months after the last infection.
\(^{c}\)Not significantly different (within the 95% confidence limits) from uninfected controls.
Table V-3. VV-Immune Mice Challenged with Heterologous Viruses

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
<th>NP-Pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV+LCMV</td>
<td>737 (586-992)^a</td>
<td>329 (259-452)</td>
<td>321 (259-424)</td>
<td>519 (417-687)^b</td>
</tr>
<tr>
<td>V+L+P+M</td>
<td>1,356 (1,039-1,955)</td>
<td>345 (261-506)</td>
<td>726 (572-993)</td>
<td>663 (521-909)</td>
</tr>
<tr>
<td>VV+LCMV</td>
<td>2,830 (2,250-3,812)</td>
<td>238 (192-311)</td>
<td>371 (307-472)</td>
<td>895 (729-1,159)</td>
</tr>
<tr>
<td>V+L+P+M</td>
<td>2,602 (2,079-3,478)</td>
<td>625 (513-800)</td>
<td>907 (754-1,141)</td>
<td>922 (766-1,156)</td>
</tr>
</tbody>
</table>

^a95% confidence limits.
^bNot significantly different (within the 95% confidence limits) from uninfected controls.
^cMice were infected with VV, LCMV, PV, and MCMV at two month intervals and sampled at least 2 months after the last infection.
in the second experiment, in comparison with VV+LCMV-immune mice. However, interpretation of this drop is more problematic for the CD4\(^+\) Thp than for the CD8\(^+\) CTLp because, as is demonstrated in all these experiments, immunization with multiple viruses results in higher background values against uninfected APC, even though each virus has been either highly diluted in PBS or purified over sucrose gradients. These results reinforce my earlier observations concerning the contribution of contaminating antigens in the virus preparations and show how even small contaminants may elicit measurable CD4\(^+\) T cell responses. Nonetheless, in contrast to the stable LCMV-specific CD4\(^+\) Thp frequency I observe in LCMV+PV+VV+MCMV- or PV+LCMV+VV+MCMV-immune mice, VV+LCMV+PV+MCMV-immune mice seem to exhibit a slight drop in the LCMV-specific CD4\(^+\) Thp frequency as compared to VV+LCMV-immune mice. These results show that, even though the CD8\(^+\) CTLp frequency to earlier infections drops significantly following multiple heterologous virus infections, the CD4\(^+\) Thp frequency remains fairly stable.

D. LCMV-specific CD4\(^+\) Thp frequency following protein antigen immunization. The above results show that the LCMV-specific CD4\(^+\) Thp, unlike the CD8\(^+\) CTLp, remain quite stable following multiple heterologous virus infections. Several reasons may account for why we observe a decline in the CD8\(^+\) CTLp and not in the CD4\(^+\) Thp frequency in this system. One possibility is that the CD4\(^+\) T cells are more
cross-reactive between these viruses than the CD8+ CTL. The activation of virus-specific memory T cells via cross-reactivity seems to protect these cells from the deletion that occurs of the memory T cells specific to earlier viruses (L.K. Selin and R.M. Welsh, unpublished observations). The participation of memory CD4+ T cells to the increased IFN-γ production following VV infection of LCMV-immune mice would seem to support this notion (Fig. V-3). In addition, in two separate experiments there were elevated LCMV-specific CD4+ Thp frequencies in PV-immune mice stimulated with LCMV-infected PEC as APC (Experiment 1:1/2,390; Experiment 2: 1/3,946) as compared to PV-immune mice stimulated with mock-infected PEC as APC (Experiment 1:1/3,956; Experiment 2: 1/8,601). I also observed a much higher LCMV-specific CD4+ Thp frequency in four of six experiments using PV+LCMV-immune mice (Fig. V-5 and Table V-2) as compared to LCMV-immune mice alone (Tables IV-1 and IV-3), suggesting that a PV infection may prime for LCMV-specific CD4+ Thp. These observations suggest that heterologous virus infections are capable of activating memory CD4+ T cells, possibly due to cross-reactivity.

Another possible reason why there is no decline in the CD4+ Thp frequency following multiple heterologous virus infections is that none of these virus infections induce as strong a CD4+ as a CD8+ T cell response. However, it should be noted, as I have shown in Chapter IV, that >10% of the CD4+ T cells score as virus-specific during infection with LCMV, a virus previously thought not to stimulate strong CD4+ T cell responses. Even
less is known about the magnitude of the CD4\(^+\) T cell response to each of the other viruses used here. In order to address this issue, I examined the LCMV-specific CD4\(^+\) Tfh frequency in LCMV-immune mice challenged with one or two consecutive protein antigens in adjuvant in an attempt to induce as strong a CD4\(^+\) T cell response as possible. Table V-4 shows that LCMV-immune mice challenged with the complex protein antigen KLH in adjuvant (CFA) does not reduce the LCMV-specific CD4\(^+\) Tfh frequency to whole virus or either of the two MHC class II-restricted peptides. Even an additional immunization with the protein antigen OVA in a different adjuvant (TiterMax Gold) failed to reduce the LCMV-specific CD4\(^+\) Tfh frequency (Table V-5). A measurable protein-antigen specific CD4\(^+\) Tfh frequency was detectable in each of these experiments, demonstrating that each of these immunizations efficiently stimulated a CD4\(^+\) T cell response. These results suggest that even in the face of a strong CD4\(^+\) T cell stimulus, the LCMV-specific CD4\(^+\) Tfh frequency remains quite stable.

E. Intracellular staining of peptide-specific IFN-\(\gamma\)-producing CD4\(^+\) T cells following multiple heterologous virus infections. In experiments presented in Chapter IV, I show that LCMV-specific CD4\(^+\) T cells can be detected following stimulation with peptide and IL-2 by staining for intracellular IFN-\(\gamma\) production and, after the virus has been cleared and the immune system returns to homeostasis, the percentage of CD4\(^+\) T cells that stain IFN-\(\gamma\) remains stable into long-term immunity. Here I
Table V-4. LCMV-Immune Mice Challenged with KLH in CFA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
<th>NP-Pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMV</td>
<td>34,156 (22,015-76,141)^a</td>
<td>1,037 (863-1,298)</td>
<td>962 (766-1,293)</td>
<td>5,029 (4,099-6,503)</td>
</tr>
<tr>
<td>L+CFA^b</td>
<td>22,906 (16,688-36,507)</td>
<td>1,107 (874-1,508)</td>
<td>1,516 (1,246-1,865)</td>
<td>8,802 (7,009-11,824)</td>
</tr>
<tr>
<td>L+KLH^c,d</td>
<td>18,940 (14,061-29,011)</td>
<td>786 (591-1,175)</td>
<td>849 (682-1,123)</td>
<td>5,775 (4,649-7,622)</td>
</tr>
<tr>
<td>LCMV</td>
<td>33,469 (23,107-60,679)</td>
<td>2,671 (2,123-3,597)</td>
<td>1,442 (1,186-1,835)</td>
<td>12,513 (9,525-18,238)</td>
</tr>
<tr>
<td>L+CFA</td>
<td>44,347 (27,980-106,849)</td>
<td>2,569 (2,076-3,370)</td>
<td>3,122 (2,540-4,051)</td>
<td>8,788 (6,971-11,884)</td>
</tr>
<tr>
<td>L+KLH^c</td>
<td>49,141 (29,384-150,005)</td>
<td>3,352 (2,607-4,691)</td>
<td>1,932 (1,617-2,397)</td>
<td>18,773 (12,517-37,528)^f</td>
</tr>
<tr>
<td>LCMV</td>
<td>24,993 (17,985-40,951)</td>
<td>893 (736-1,135)</td>
<td>553 (444-733)</td>
<td>4,735 (3,834-6,191)</td>
</tr>
<tr>
<td>L+CFA</td>
<td>14,469 (11,310-20,075)</td>
<td>1,017 (812-1,360)</td>
<td>996 (829-1,246)</td>
<td>4,007 (3,245-5,235)</td>
</tr>
<tr>
<td>L+KLH^f</td>
<td>26,679 (18,938-45,120)</td>
<td>1,048 (849-1,366)</td>
<td>1,796 (1,431-2,411)</td>
<td>6,298 (5,022-8,444)</td>
</tr>
</tbody>
</table>

^a95% confidence limits.
^bLCMV-immune mice (L, 2 months) were immunized with complete Freund's adjuvant (CFA) as a control.
^cLCMV-immune mice (2 months) were immunized with the protein antigen keyhole limpet hemocyanin (KLH) emulsified in complete Freund's adjuvant and sampled 2 months later.
KLH-APC yielded a frequency of: 1,965 (1,613-2,514).
KLH-APC yielded a frequency of: 1,829 (1,437-2,516).
Not significantly different (within the 95% confidence limits) from uninfected controls.
KLH-APC yielded a frequency of: 1,709 (1,402-2,191).
Table V.5. LCMV-Immune Mice Challenged with KLH in CFA and OVA in Titer Max Adjuvant

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
<th>NP-Pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMV</td>
<td>3,592 (2,954-4,581)(^a)</td>
<td>211 (174-270)</td>
<td>475 (369-666)</td>
<td>4,339 (3,053-7,501)(^b)</td>
</tr>
<tr>
<td>L+CFA+TMG(^c)</td>
<td>5,643 (4,582-7,342)</td>
<td>347 (260-521)</td>
<td>706 (547-996)</td>
<td>7,227 (5,118-12,288)(^b)</td>
</tr>
<tr>
<td>L+KLH+OVA(^d,e)</td>
<td>22,959 (13,714-70,453)</td>
<td>407 (319-561)</td>
<td>603 (452-907)</td>
<td>6,361 (4,554-10,539)</td>
</tr>
<tr>
<td>LCMV</td>
<td>6,118 (4,802-8,429)</td>
<td>907 (724-1,214)</td>
<td>900 (749-1,127)</td>
<td>15,840 (10,961-28,547)(^b)</td>
</tr>
<tr>
<td>L+ CFA+TMG</td>
<td>12,091 (8,864-19,010)</td>
<td>426 (351-543)</td>
<td>1,042 (902-1,234)</td>
<td>9,060 (6,693-14,016)(^b)</td>
</tr>
<tr>
<td>L+ KLH+OVA(^f)</td>
<td>9,966 (7,541-14,694)</td>
<td>414 (340-528)</td>
<td>562 (459-724)</td>
<td>10,207 (7,323-16,836)(^b)</td>
</tr>
</tbody>
</table>

\(^a\)95% confidence limits.
\(^b\)Not significantly different (within the 95% confidence limits) from uninfected controls.
\(^c\)LCMV-immune mice (L, 2 months) were immunized with complete Freund’s adjuvant (CFA) followed by TiterMax Gold (TMG) adjuvant at two month intervals as a control.
\(^d\)LCMV-immune mice (2 months) were immunized with the protein antigens keyhole limpet hemocyanin (KLH) emulsified in complete Freund’s adjuvant and chicken ovalbumin (OVA) at two month intervals and sampled 2 months after the last immunization.
\(^e\)KLH-APC yielded a frequency of: 1,556 (1,196-2,226); OVA-APC yielded a frequency of: 3,724 (2,822-5,477).
\(^f\)KLH-APC yielded a frequency of: 899 (728-1,177); OVA-APC yielded a frequency of: 5,274 (4,152-7,226).
questioned if the frequency of LCMV-specific IFN-γ-producing CD4+ T cells also remain stable following heterologous virus infections, as I have just demonstrated above using the IL-2-based CD4 LDA. In several experiments, all done using some of the same mice utilized above in the CD4 LDA, the intracellular IFN-γ assay mirrored the results of the IL-2-based CD4 LDA, except that the frequencies of antigen-specific cells were in all cases much higher for reasons already discussed in Chapter IV (see Table V-6). Thus, for LCMV+PV+VV+MCMV- and PV+LCMV+VV+MCMV-immune mice, there was no significant reduction in the frequency of CD4+ T cells that produced IFN-γ following stimulation with either of the two LCMV MHC class II-restricted CD4 peptides. Interestingly, as the LDA results suggested above, I did observe a slight, but significant ($p<0.05$), 2-fold reduction in the frequency of CD4+ T cells that produced IFN-γ in VV+LCMV+PV+MCMV-immune mice as compared to VV+LCMV-immune mice (Table V-6). These results support the CD4 LDA results discussed above suggesting that the LCMV-specific CD4+ Thp frequency remains relatively stable following multiple heterologous virus infections. These results also clearly demonstrate that, even though the intracellular IFN-γ assay detects a higher frequency of virus-specific CD4+ T cells, it mirrors any differences observed in the virus-specific CD4+ Thp frequencies obtained using the IL-2-based CD4 LDA.
Table V-6. Frequency of IFN-γ-Producing Peptide-Specific CD4⁺ T cells Following Heterologous Virus Infections

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage of IFN-γ⁺ CD4⁺ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GP61-80</td>
</tr>
<tr>
<td>LCMV Imm</td>
<td>0.76±0.43</td>
</tr>
<tr>
<td>LCMV+PV+VV+MCMV</td>
<td>0.83±0.68</td>
</tr>
<tr>
<td>PV+LCMV Imm</td>
<td>1.07±0.38</td>
</tr>
<tr>
<td>PV+LCMV+VV+MCMV</td>
<td>1.20±0.40</td>
</tr>
<tr>
<td>VV+LCMV Imm</td>
<td>1.36±0.58</td>
</tr>
<tr>
<td>VV+LCMV+PV+MCMV</td>
<td>0.70±0.36⁺</td>
</tr>
</tbody>
</table>

Splenocytes from immune C57BL/6 mice were stimulated with one of the two LCMV MHC class II-restricted peptides in the presence of IL-2 and brefeldin A for 5 hr as described in the Materials and Methods. The numbers shown represent the percentage of CD4⁺IFN-γ⁺ T cells in the spleen. Means ± standard deviations for times points from 1-2 separate experiments with 3 individual mice per experiment are shown (n=3-6 per group).

⁺Significantly different as compared to VV+LCMV-Immune mice, p<0.05.
F. Intracellular staining of cytokine-producing CD4$^+$ T cells following acute LCMV, PV, VV, or MCMV infection. As mentioned above, one reason for the observed differences in the stability of the CD8$^+$ versus the CD4$^+$ T cells following heterologous virus infections may be the degree to which each virus activates CD4$^+$ T cells as compared to CD8$^+$ T cells. Each of these viruses are known inducers of CD8$^+$ CTL. In order to examine the extent to which each of these viruses activate CD4$^+$ T cells, I performed intracellular IFN-γ staining on CD4$^+$ T cells following PMA and ionomycin activation from naive mice challenged with each of the four viruses used in these experiments. As can be seen in Figure V-6, each of the viruses stimulates increased frequencies of CD4$^+$ T cells capable of making IFN-γ when nonspecifically stimulated with PMA and ionomycin. This, combined with the upregulation of activation markers discussed earlier in this chapter, suggests that each of these viruses are capable of activating a virus-specific CD4$^+$ T cell response. However, it is difficult to accurately measure the relative magnitude of the virus-specific CD4$^+$ T cell response to PV, VV, or MCMV without having any of the MHC class II-restricted CD4 epitopes.

G. Brief summary to Chapter V. Together, the data presented in this chapter show that memory CD4$^+$ T cells are capable of participating in heterologous T cell-mediated immunity through the increased production of cytokines such as IFN-γ following a heterologous virus infection (Selin et al., 1998). However, unlike CD8$^+$ T cells, CD4$^+$
Figure V-6. Intracellular IFN-γ expression of virus-induced CD4+ T cells following stimulation with PMA and ionomycin. Splenocytes from uninfected (day 0), or infected with LCMV, VV, PV, or MCMV for 9 days were stimulated with PMA and ionomycin for 2 hr before the addition of brefeldin A for an additional 2 hr as described in the Materials and Methods. The numbers shown indicate the percentage of CD4+ T cells that stained positive for intracellular IFN-γ as represented by the R1 gate. Data are representative of 2 experiments with 2 individual mice per experiment (n=4 per group).
Thp frequency is resistant to reduction by heterologous virus infections, even under conditions that reduce the LCMV-specific CD8+ CTLp frequency (Selin et al., 1996).
CHAPTER VI

EXHAUSTION OF LCMV-SPECIFIC CD4+ T CELLS
FOLLOWING HIGH DOSE LCMV INFECTION

The last two chapters provide evidence that the LCMV-specific CD4+ Th frequency remains extremely stable even under conditions that reduce the LCMV-specific CD8+ CTLp frequency. Infection of mice with a high-dose of LCMV Clone 13, a highly disseminating variant capable of rapidly growing to high titers in all of the peripheral organs, results in the transient activation, followed by exhaustion and clonal deletion of the virus-specific CD8+ CTL under the stress of the overwhelming viral antigen environment (Moskophidis et al., 1993). The fate of the virus-specific CD4+ T cells under these conditions is not known. In this chapter I followed the fate of the LCMV-specific CD4+ T cells using LDA and intracellular IFN-γ staining following peptide stimulation. I will show that, like the CD8+ CTL, there is a decline in the frequency of detectable virus-specific CD4+ T cells following a high-dose LCMV infection.

A. Quantitation of the CD4+ Thp frequency following LCMV Clone 13 infection. Table VI-1 shows an analysis of the LCMV-specific CD4+ Thp frequency of C57BL/6 mice following a high-dose LCMV Clone 13 infection. The LCMV-specific
Table VI-1. CD4+ T Cell Precursor Frequencies Following LCMV Clone 13 Infection

<table>
<thead>
<tr>
<th>Groups</th>
<th>Uninfected APC</th>
<th>LCMV-infected APC</th>
<th>GP-Pulsed APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 10 Armstrong</td>
<td>21,992 (15,847-35,923)</td>
<td>598 (493-760)</td>
<td>142 (110-202)</td>
</tr>
<tr>
<td>Day 7 Clone 13</td>
<td>9,164 (6,206-17,504)</td>
<td>3,872 (2,786-6,347)</td>
<td>4,881 (3,712-7,125)</td>
</tr>
<tr>
<td>Day 10 Clone 13</td>
<td>38,287 (23,531-102,652)</td>
<td>750 (595-1,014)</td>
<td>273 (216-367)</td>
</tr>
<tr>
<td>Day 15 Clone 13</td>
<td>7,337 (5,704-10,279)</td>
<td>4,851 (3,920-6,360)</td>
<td>4,520 (3,674-5,873)</td>
</tr>
</tbody>
</table>

*a95% confidence limits.

*bNot significantly different (within the 95% confidence limits) from uninfected controls.
CD4+ Thp frequency peaked at day 10 p.i. at a frequency comparable to that obtained after a low-dose LCMV Armstrong infection. However, unlike my previous results with LCMV Armstrong (see Table IV-1), the virus-specific CD4+ Thp frequency declined to undetectable levels by day 15 p.i. with LCMV Clone 13. These results suggest that high-dose LCMV Clone 13 infection induces the exhaustion of the CD4+ T cells.

B. Intracellular staining of peptide-specific IFN-γ-producing CD4+ T cells following LCMV Clone 13 infection. Using the IL-2-based LDA, the above results suggest that a transient virus-specific CD4+ T cell response occurs following a high-dose LCMV Clone 13 infection. I utilized the intracellular IFN-γ assay described in Chapter IV to follow the fate of the IFN-γ-secreting CD4+ T cells under these same conditions. Figure VI-1 shows the percentage of peptide-specific IFN-γCD4+ T cells using the same mice used in the LDA above. The percentage of IFN-γCD4+ T cells never attained the percentage observed during LCMV Armstrong infection, but interestingly, the frequency of IFN-γ-secreting cells induced by Clone 13 does not decline below detectable levels, as observed above for the IL-2-producing cells measured in the LDA. Closer examination of these stains revealed that all of the CD4+ T cells that stained brightest for IFN-γ were absent in the LCMV Clone 13-infected animals (Fig. VI-2). Analysis of several activation markers on CD4+ T cells demonstrated that there was increased expression of TCR-
Figure VI-1. Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells following LCMV Clone 13 or Armstrong infection. Splenocytes from the indicated days p.i. were stimulated with one of two LCMV MHC class II-restricted peptides in the presence of IL-2 and brefeldin A for 5 hr, as described in the Materials and Methods. Background staining with the appropriate isotype-matched control mAb was subtracted from each individual. Means ± standard deviations for time points from one of three similar experiments with 3 individual mice per experiment are shown (n=3 per group).
Figure VI-2. Intracellular IFN-γ expression of LCMV peptide-specific CD4+ T cells from LCMV Clone 13-infected C57BL/6 mice. Splenocytes from LCMV Armstrong- or Clone 13-infected mice (both day 10) were stimulated with the LCMV MHC class II-restricted peptide GP61-80 in the presence of IL-2 and brefeldin A for 5 hr as described in the Materials and Methods. The numbers shown indicate the percentage of CD4+ T cells that stained positive for intracellular IFN-γ as represented by the R1 gate. Data are representative of 3 experiments.
Table VI-2. Expression of Activation Markers on CD4⁺ T cells During the Acute LCMV Clone 13 Infection

<table>
<thead>
<tr>
<th>Day P.I.</th>
<th>CD25&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>CD44&lt;sup&gt;hi&lt;/sup&gt;</th>
<th>CD62L&lt;sup&gt;lo&lt;/sup&gt;</th>
<th>CD69&lt;sup&gt;hi&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>10± 2</td>
<td>16± 3</td>
<td>16± 7</td>
<td>9± 3</td>
</tr>
<tr>
<td>Day 10 Armstrong</td>
<td>11± 2</td>
<td>37± 5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>47± 9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>11± 3</td>
</tr>
<tr>
<td>Day 10 Clone 13</td>
<td>14± 1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>32± 4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>49± 4&lt;sup&gt;*&lt;/sup&gt;</td>
<td>19± 4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 15 Clone 13</td>
<td>16± 2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>43± 3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>47± 7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>26± 7&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 21 Clone 13</td>
<td>17± 2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>38± 7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>41± 5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>26± 4&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Spleen cells were from mice either uninfected (day 0), infected with LCMV Armstrong (day 10), or LCMV Clone 13 for 10, 15, or 21 days. Cells were stained for expression of CD4 and various activation markers as described in the Materials and Methods. Means ± standard deviations for time points from 2 separate experiments with 3 individual mice per experiment are shown (n=6 per group).

*Indicates a significant difference as compared to day 0, p<0.05.
dependent markers such as CD25 and CD69 throughout the time points examined (Table VI-2). This demonstrates that, even though the IL-2-producing CD4⁺ Thp cannot be detected, CD4⁺ T cells expressing activation markers are still present in vivo and have not been deleted.

Annexin V staining of CD4⁺ T cells was performed to examine whether there is an increased frequency of CD4⁺ T cells undergoing apoptosis following LCMV Clone 13 expression. One of the earliest events in apoptosis is the translocation of the membrane phospholipid phosphatidylserine to the outside of the plasma membrane. Annexin V is a Ca²⁺-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine. Table VI-3 shows that, there is a higher percentage of apoptotic CD4⁺ T cells during LCMV Clone 13 infection as compared to naive mice at all time points tested. Thus, unlike LCMV Armstrong infection, in which there is a peak in the percentage of CD4⁺ T cells undergoing apoptosis once virus has been cleared, LCMV Clone 13 infection seems to induce a steady rate of apoptosis in the CD4⁺ T cell population.

There are several reasons that might explain why the LDA fails to detect any virus-specific CD4⁺ Thp after day 15 pi. yet the intracellular stain can still score virus-specific CD4⁺ T cells capable of making IFN-γ out to day 15 p.i. One reason is that the two assays may be detecting mutually exclusive cell populations. Alternatively, as a result of the high antigen environment, all of the CD4⁺ T cells may be driven into terminal effector
Table VI-3. Increase in the Percentage of Apoptotic CD4\(^+\) T cells During the Acute LCMV Infection with either Armstrong or Clone 13\(^a\)

<table>
<thead>
<tr>
<th>Day Post-Infection</th>
<th>Annexin(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>Day 7 Armstrong</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>Day 9 Armstrong</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Day 11 Armstrong</td>
<td>18 ± 7</td>
</tr>
<tr>
<td>Day 15 Armstrong</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>LCMV Armstrong Immune</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Day 0</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Day 10 Armstrong</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Day 10 Clone 13</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Day 15 Clone 13</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Day 21 Clone 13</td>
<td>16 ± 4</td>
</tr>
</tbody>
</table>

\(^a\)Spleen cells were from mice either uninfected (day 0), infected with LCMV Armstrong, or LCMV Clone 13 for the indicated number of days. Cells were stained for expression of CD4 and annexin V. The numbers shown represent the percentage of CD4\(^+\) T cells that were also annexin V\(^+\). Means ± standard deviations for time points from 2 separate experiments with 3 individual mice per experiment are shown (n=6 per group).
cells in which all of the cells are producing effector cytokines such as IFN-\(\gamma\) with little production of IL-2. Another possibility is that all of the high-affinity virus-specific CD4\(^+\) T cells have been exhausted and what remains are low-affinity LCMV-specific CD4\(^+\) T this would be consistent with the weakly staining IFN-\(\gamma\)^+ cells. Finally, it may be that the CD4\(^+\) T cells may be anergic by day 15 p.i. and that the LDA, based on the production of IL-2, fail to detect them, but the intracellular IFN-\(\gamma\) assay, in which anergy may be broken by the addition of exogenous IL-2 to assay, would be able to detect anergic cells. Anergic T cells can be rescued by the addition of exogenous IL-2 in the presence of antigen.

**C. Brief summary to Chapter VI.** I have shown in this chapter that a high-dose LCMV infection alters the virus-specific CD4\(^+\) T cell response. I have provided evidence that there is a decline in the detectable frequency of IL-2-producing CD4\(^+\) Thp as detected using the CD4 LDA. In addition, I show that a large percentage of the CD4\(^+\) T cells continue to express activation markers following LCMV Clone 13 infection and that some of these CD4\(^+\) T cells are still capable of making IFN-\(\gamma\) following stimulation with peptide in the presence of IL-2. These results suggest that the CD4\(^+\) T cells may become anergic following a high-dose LCMV Clone 13 infection and are thus impaired in their ability to produce IL-2 but can be rescued to make IFN-\(\gamma\) in the presence of peptide and exogenous IL-2. This interpretation would agree with a recent study demonstrating that
LCMV-specific transgenic CD4+ T cells persist in vivo in an anergic state for >6 weeks following high-dose LCMV Docile infection (Oxenius et al., 1998).
CHAPTER VII

DISCUSSION

I have made several novel observations in this thesis concerning the magnitude, longevity, and stability of the CD4+ T cell response during an acute virus infection and into long-term memory. In addition to these findings, I have provided evidence that CZ-1, a new mAb created in our laboratory (Brutkiewicz et al., 1993; Vargas-Cortes et al., 1992), represents a novel activation and memory marker on CD4+ T cells. I have also shown that, in contrast to the conclusion of recent reports (Su et al., 1998; Whitmire et al., 1998), LCMV infection induces primarily a Th1 response characterized by increased production of IL-2 and IFN-γ by the virus-specific CD4+ T cells. Finally, I provide data that suggest that the CD4+ T cells become unresponsive following a high-dose LCMV Clone 13 infection. Taken together, the data presented in this thesis make several important observations that help provide a better understanding of virus-specific CD4+ T cell responses. Below is an in depth discussion of the major points made in this thesis.

A. Activation phenotype of CD4+ T cells and characterization of mAb CZ-1. As I detailed in the introduction, T cell activation induces many changes in naive T cells, including alterations in the expression of several cell surface molecules. Most of these
markers are expressed on both activated and memory T cells. One specific aim of this thesis was to determine the magnitude of the virus-specific CD4⁺ T cell response following an acute virus infection. I took advantage of the well-studied LCMV system to begin to address this issue, as well as pose other fundamental, yet unresolved, questions concerning virus-specific CD4⁺ T cell responses. To begin to examine the magnitude of the CD4⁺ T cell response following LCMV-infection, I utilized a panel of cell surface markers to define activated CD4⁺ T cells and showed that by day 10 p.i. with LCMV there was an increase in the cell surface expression of activation markers such as CD44, CD49d, LFA-1, and CZ-1 on gated CD4⁺ T cells (Figs. III-2, III-3, and Table III-1). Even though there was little increase in the absolute numbers of CD4⁺ T cells in the spleen expressing these activation markers, there was an increase in the relative proportion of CD4⁺ T cells expressing these activation markers (Figs. III-2 and III-3). This is most likely because there is little to no expansion in the total number of CD4⁺ T cells in the spleen during an acute LCMV infection (Fig. III-1). A similar increase in the proportion of CD4⁺ T cells expressing elevated cell surface levels of CD49d and CD62L in the spleen following infection with the Traub strain of LCMV in Balb/c mice has been previously described (Andersson et al., 1994). However, the percentage of CD4⁺ T cells that remain in the spleen following infection with this strain of LCMV is even lower (6%) than what I have observed here using LCMV Armstrong (10%, Fig. III-1). More recent studies using LCMV Armstrong have reported similar percentages of CD4⁺ T cells
expressing CD44 than what I have shown here (Whitmire et al., 1998). I also show that 25% of the CD4+ T cells in the spleen were blast-sized at day 7 p.i. and that 7% of the CD4+ T cells were in the G2+M or S phase of the cell cycle. Again, these data contrast with a previous report demonstrating that only 2% of CD4+ T cells exhibited increased DNA content following an acute infection with the Traub strain of LCMV in Balb/c mice (Christensen et al., 1996). The discrepancy between these findings and mine may be due to differences in the infecting dose of virus, strains of virus, or strains of mice that were used in these studies. My results clearly show that a large proportion of the CD4+ T cells express an activated cell phenotype following an acute LCMV infection.

Interestingly, the peak of expression of the very early activation antigen CD69 occurred at 2-3 days p.i. (Fig. III-4 and data not shown), well before the peak of the other markers discussed above (Table III-1 and Fig. III-4). This expression pattern parallels the type I IFN response in these mice (Leist et al., 1987). To my knowledge, type I IFN has not been previously reported to upregulate CD69 expression on CD4+ T cells, though it has been shown to induce CD69 expression on NK cells (Testi et al., 1994). However, because I have not directly measured the amount of type I IFN in these animals, more studies would need to be performed in order to establish that type I IFN can upregulate CD69 expression on CD4+ T cells.

I present data in this thesis that shows that the sialated CD45RB-associated epitope, detected by mAb CZ-1 is both an activation and a memory marker on virus-induced
CD4+ T cells. MAb CZ-1 identifies an antigen that appears late in the differentiation of CD4+ T cells. It reacts with only 1% of the CD4+CD8- thymocytes (Vargas-Cortes et al., 1992), and in contrast to CD8+ T cells, the CZ-1 epitope is expressed on only a small proportion of mature CD4+ T cells. However, its expression is increased on most activated CD4+ T cells, including all of the IL-2-responsive CD4+ T cells (Brutkiewicz et al., 1993). Here I show that the mAb CZ-1 heterogeneously stains CD4+ T cells in a pattern distinct from that exhibited by anti-CD45RB (Ernst et al., 1990). When CD4+ T cells from the spleens of mice undergoing an acute infection with LCMV were stained with both mAb CZ-1 and anti-CD45RB, a subpopulation of CZ-1+CD45RBlo cells appeared (Fig. III-6). Further analyses of the cell surface phenotype during the acute infection with LCMV revealed that the blast-sized CD4+ T cells were CZ-1+CD45RBb+CD44hiCD62L- (Fig. III-7 and III-8). Therefore, the CZ-1 antigen was upregulated upon CD4+ T cell activation. Since CZ-1 recognizes a sialic acid-associated epitope of CD45RB, CD4+ T cells may begin sialating CD45RB molecules upon activation. The increase in a CD45RB-associated epitope would seem at odds with the observation that memory CD4+ T cells express lower levels of surface CD45RB (Birkeland et al., 1989), but a conversion from non-sialated to sialated forms of the molecule could account for the CZ-1 staining patterns, even though the total protein levels may be reduced.
Experiments on the function of CD4⁺ T cell subsets separated by CZ-1 indicate that this antibody identifies a functionally distinct population of T cells. The CD4⁺CZ-1⁺ T cells contained all of the LCMV-specific memory. It was also shown that no memory could be rescued from the CD4⁺CZ-1⁻ population, even in the presence of exogenous IL-2 (Fig. III-11). CZ-1 expression can be upregulated by anti-CD3 or concanavalin A in non-expressing CD4⁺ T cells and is expressed on all IL-2-responsive CD4⁺ T cells (Brutkiewicz et al., 1993; Vargas-Cortes et al., 1992). Currently, antibodies to CD45RB are commonly used to distinguish naive and antigen primed/memory CD4⁺ T cells (Bradley et al., 1993; Gray, 1993; Lee et al., 1990). However, unlike CD45RB, CZ-1 is a positive marker for CD4⁺ T cell memory in mice. In the LDA studies examining the phenotype of the memory CD4⁺ Thp, CZ-1 was better than most of the markers used, and at least as good as CD49d, for detecting LCMV-specific memory CD4⁺ T cells (Table IV-4). Taken together, these data show that CZ-1 represents a novel activation and memory marker on murine CD4⁺ T cells.

B. Analysis of the cytokine response during LCMV infection. Another specific aim of this thesis was to determine the cytokine profile induced following LCMV infection. Recent work in several virus models, including LCMV, influenza virus, Sendai virus, MHV-68, and HIV infection in humans has suggested that a mixture of cells secreting type 1 and type 2 cytokine profiles are induced by the viral infection (Alonso et al., 1997; Carding et al., 1993; Meyaard et al., 1994; Mo et al., 1995; Sarawar et al., 1996;
Sarawar and Doherty, 1994; Su et al., 1998; Whitmire et al., 1998). However, few of these studies have examined the relative contribution of CD4+ and CD8+ T cells to this cytokine production following an acute virus infection. Thus, there is little information concerning the cytokine profile of CD4+ T cells that are induced following a virus infection. In this thesis I have examined cytokine production using several different techniques including ELISA, ELISPOT assays, and new methods based on intracellular cytokine staining following in vitro peptide stimulation. The latter assay in particular allowed me to examine both the type of cytokine-secreting cells elicited following LCMV infection as well as to determine the magnitude of the virus-specific CD4+ T cell response. Using ELISPOT assays to detect individual cytokine-secreting cells, I demonstrate that the frequency of IFN-γ-secreting cells in the spleen peaked by day 9 p.i., in general agreement with previous work by Gessner et al. using similar assays following LCMV WE infection (Fig. III-14 and (Gessner et al., 1990; Gessner et al., 1989). The frequency of IFN-γ-producing splenocytes at day 7 p.i. was 10-fold higher than that reported by Cousens et al. using the same strain of mice and virus, but her assays were done in the absence of LCMV-infected APC (Cousens et al., 1995). Interestingly, the frequencies of IFN-γ- and IL-4-producing cells reported by this group are almost identical to those shown in Table III-2 using virus contaminated with FCS antigens (Cousens et al., 1995; Su et al., 1998). In addition, I have failed to detect any significant IL-4 production following assay of conditioned media or following in vitro
restimulation with the LCMV MHC class II-restricted CD4 peptides. This suggests that some of the recent reports of Th2 responses during LCMV infection may be inaccurate due to FCS antigens present in the virus preparations (Su et al., 1998; Whitmire et al., 1998). This idea is further supported by later experiments showing that no LCMV-specific CD4+ Thp could be detected by LDA using the very IL-4-sensitive CT.4S cell line. Furthermore, intracellular cytokine staining following polyclonal stimulation with PMA and ionomycin revealed no increase in the frequency of IL-4-producing cells during an acute LCMV infection (Fig. IV-2). Likewise, I could detect no increase in intracellular IL-4, IL-5, or IL-10 levels following LCMV infection, even in mice genetically deficient in CD8+ T cells that mount a very strong virus-specific CD4+ T cell response following LCMV infection (Fig. IV-6). Work by other groups have failed to detect increases in IL-4 production following LCMV infection, including a recent study using a transgenic CD4+ T cell specific for the GP61-80 epitope of LCMV (Homann et al., 1998; Oxenius et al., 1998; Oxenius et al., 1996). Thus, LCMV infection induces cytokine production that is consistent with a polarized Th1 phenotype. It remains to be seen whether the IL-4 production reported in other virus systems is also the result of T cell responses to nonviral antigens as I have shown here for LCMV.

The above experiments I performed did not address what cells were responsible for making the IFN-γ. FACS-purification of CD4+ and CD8+ T cells revealed that there were consistently more CD8+ than CD4+ T cells secreting IFN-γ cells during the acute LCMV
infection, as detected at the single-cell level utilizing the ELISPOT assay. This ratio is not surprising, given that later experiments will demonstrate that there are approximately 7- to 10-fold more CD8+ CTLp than CD4+ Thp at these time points during the acute LCMV infection (Table IV-1 and (Selin et al., 1996)). Interestingly, in 4 independent experiments, the CD4+ and CD8+ T cells secreted comparable amounts of IFN-γ protein into the supernatant even though there were many more IFN-γ-secreting CD8+ T cells than CD4+ T cells as detected by ELISPOT assays (Table III-3). This observed difference may be explained by fact that LCMV infection induces CD8+ CTL with high cytotoxic activities that may be capable of destroying virus-infected APC and thus reducing the amount of IFN-γ that is produced by the CD8+ CTL (Planz et al., 1996). However, this seems unlikely given that the number of LCMV-infected APC used in these assays is in great excess over the frequency of responding CD8+ T cells. Of interest is that recent studies have found that CD8+ CTL clones with high cytotoxic activity produced low amounts of cytokines (Sad et al., 1996). Thus, these results suggest that CD4+ T cells make more IFN-γ on a per cell basis, than CD8+ T cells during an acute LCMV infection. This conclusion is also consistent with the relative intensity of IFN-γ staining we observe in the intracellular IFN-γ assay using either CD4+ or CD8+ T cells. In this assay, the CD4+ T cells often demonstrate a higher intensity of IFN-γ staining that
the CD8\(^+\) T cells. Taken together, these data show that LCMV infection induces CD4\(^+\) T cells with predominantly a Th1 phenotype.

**C. Detection of a high frequency of LCMV-specific CD4\(^+\) T cells.** Another specific aim of this thesis was to determine the magnitude of the LCMV-specific CD4\(^+\) T cell response following acute LCMV infection. Examination of the CD4\(^+\) Thp frequency using the IL-2-based LDA during an acute LCMV infection and on into the memory state revealed that within 10 days p.i. with LCMV, the CD4\(^+\) Thp frequency rose from <1/100,000 to approximately 1/600 (Table IV-1). Quantitation of virus-specific CD4\(^+\) Thp frequencies using LDA in several other virus systems has yielded CD4\(^+\) Thp frequencies that range from 1/300 to 1/2000 from the peak of the acute response into long-term memory (Ewing et al., 1995; Topham and Doherty, 1998; Topham et al., 1996; Tripp et al., 1997). Thus, even though 25\% of the CD4\(^+\) T cells are blast-sized at day 7 p.i. and 25-50\% of the CD4\(^+\) T cells express activation markers, <1\% of these cells scored in LDA as being virus-specific. Using new sensitive assays to measure IFN-\(\gamma\) production at the single-cell level, I show that >20\% of the CD4\(^+\) T cells secreted IFN-\(\gamma\) after polyclonal stimulation with PMA and ionomycin (Fig. IV-2), and >10\% of the CD4\(^+\) T cells secreted IFN-\(\gamma\) after stimulation with either of the two LCMV MHC class II-restricted peptides (Fig. IV-5). The total number of virus-specific CD4\(^+\) T cells may be even higher than I show here, since only two CD4 peptides were used. It is likely that
the CD4$^+$ T cell response to LCMV includes other MHC class II-restricted epitopes which have yet to be identified. Thus, these new sensitive assays reveal a previously unappreciated profound antigen-specific CD4$^+$ T cell response during viral infections.

Because LDA could only account for a few percent of the T cells as being virus-specific (Asano and Ahmed, 1996; Butz and Bevan, 1998; Murali-Krishna et al., 1998; Selin et al., 1996; Varga and Welsh, 1998), it had been suggested that bystander activation of the T cells by cytokines released during the virus infection might account for the vast majority of the responding T cells (Tough et al., 1996; Tough and Sprent, 1996). However, the data presented here, in conjunction with recent studies examining the specificity of the CD8$^+$ T cells (Butz and Bevan, 1998; Murali-Krishna et al., 1998; Zarozinski and Welsh, 1997), argue that most of the T cells being activated during an acute LCMV infection are indeed virus-specific.

D. Stability of LCMV-specific CD4$^+$ Thp into long-term immunity. Determining the longevity of CD4$^+$ T cell memory following an acute virus infection was another specific aim of this thesis. Again I have made use of several different assays to examine this important issue. Using CD4 LDA based on IL-2-production, I show that the frequency of LCMV-specific CD4$^+$ T cells within the CD4$^+$ T cell pool remains remarkably constant from the peak in the acute viral infection and throughout long-term memory. Using this assay, the CD4$^+$ Thp frequencies one year after infection are only 2-
fold lower than their peak at day 10 p.i. This result mirrors recent work from our laboratory that has shown that the frequency of LCMV-specific CTLp per CD8+ T cell drops only 2-fold from its peak, as the acute LCMV infection converts into a memory state (Selin et al., 1996). This 2-fold drop per CD8+ T cell occurs during the more global 5 to 10-fold drop in the total CD8+ T cell number per spleen, as the immune response silences and the CD8/CD4 ratio converts from 2:1 to 1:2, as shown in Figure III-1. A similar observation has been made in an LCMV-specific CD8+ TCR transgenic model, in which the frequency of adoptively transferred transgenic T cells reached 70% of the CD8+ T cells during the peak of the acute infection and remained at 27% in the memory state (Zimmermann et al., 1996). However, I observe a slightly larger 2- to 7-fold drop in the CD4+ T cell frequency when peptides are used as the stimulus in the CD4 LDA (Table IV-3). Likewise, using the intracellular IFN-γ assay, the percentage of CD4+ T cells that are LCMV peptide-specific declines 5- to 7-fold from the peak of the response into long-term immunity (Fig. IV-5). This agrees with the results I obtained in the LDA using these two peptides but contrasts with the consistent 2-fold drop I observe in the LDA using whole virus as discussed above. This discrepancy may be due to the detection of lower affinity T cells following peptide stimulation as compared to using whole virus.

Studies following the fate of protein antigen-specific transgenic CD4+ T cells have observed large 20- to 40-fold reductions in the total number of transgenic CD4+ T cells following protein antigen immunization as the immune system returns to homeostasis.
Recent work in the influenza and Sendai virus systems have also reported larger 5- to 10-fold reductions in the virus-specific CD4\(^+\) Thp from the peak of the acute response into memory (Doherty et al., 1996; Ewing et al., 1995; Topham et al., 1996). In addition, recent studies examining LCMV-specific CD8\(^+\) T cell frequencies using MHC class I tetramers loaded with immunodominant viral peptides or intracellular IFN-\(\gamma\) staining following viral peptide stimulation have also shown approximately 6-fold reductions in the virus-specific CD8\(^+\) T cell frequency from the peak of the acute response into memory (Butz and Bevan, 1998; Murali-Krishna et al., 1998). Thus, studies performed in a variety of systems have reported variable drop-offs in the frequency of antigen-specific T cells from the peak of the response into memory.

It is currently unclear why these new assays detect such a higher frequency of virus-specific T cells than can be detected using LDA. The efficiency of the LDA is understood to be less than 100%, and it may be that LDA vastly underestimate the frequency of virus-specific T cells at the peak of the acute virus infection. In the case of the LDA for CD8\(^+\) CTLp, this assay requires the T cell to not only proliferate under \textit{in vitro} stimulation conditions, but then to kill virus-infected target cells. Thus, this assay would fail to score any virus-specific CD8\(^+\) T cell that is not cytotoxic, such as virus-specific CD8\(^+\) T cells that may be involved in predominantly secreting cytokines as opposed to killing infected cells. Thus, this may account for why staining of T cells using these other techniques based on cytokine production or TCR specificity yields a much
higher frequency of virus-specific T cells than the LDA (Butz and Bevan, 1998; Murali-Krishna et al., 1998). As for the CD4 LDA, a similar problem may exist, as stimulation with the CD4 peptides may be able to activate lower affinity virus-specific CD4⁺ T cells that are not detected by stimulation with the whole virus. However, even though I observe a slightly larger drop in the virus-specific CD4⁺ Thp frequency following peptide stimulation, once virus has been cleared, both the IL-2-based LDA and the intracellular IFN-γ assay reveal that the LCMV peptide-specific CD4⁺ T cell frequency remains remarkably stable in the memory state for essentially the life-time of the mouse (Table IV-1, Table IV-3, and Fig. IV-5).

**E. Role of CD4⁺ T cells during heterologous virus infections.** Ongoing work in our laboratory suggests that memory T cells can be important mediators in the early immune response of immune mice challenged with a heterologous virus (Selin et al., 1998; Welsh et al., 1996). Putatively unrelated viruses that do not normally induce cross-reactive CD8⁺ CTL responses will elicit cross-reactive responses if mice immune to one virus are challenged with another. Presumably a subset of memory CD8⁺ CTL clones specific to the first virus cross-reacts sufficiently with the second virus to be stimulated and amplified in number (Selin et al., 1994; Selin and Welsh, 1994). A specific aim of this thesis was to determine if memory CD4⁺ T cells could play a role in contributing to heterologous T cell immunity. I demonstrate that CD4⁺ T cells are also capable of
responding early (day 3) during a heterologous viral infection by secreting IFN-γ (Figs. V-2 and V-3). Our laboratory has reported that early memory CD8$^+$ T cell responses can mediate a reduction in virus replication following challenge with heterologous viruses (Welsh et al., 1996), but work performed more recently in collaboration with Dr. L.K. Selin suggests that this protection may involve the combined efforts of both memory CD4$^+$ and memory CD8$^+$ T cells (Selin et al., 1998). It has been shown that memory T cells are capable of secreting high quantities of cytokines (Bradley et al., 1993). Thus, one possible mechanism by which CD4$^+$ T cells may act early during a heterologous viral infection is through the secretion of cytokines such as IL-2, IFN-γ, or TNF-α, which could in turn activate and amplify the number of cross-reactive memory CD8$^+$ CTL to lyse virus-infected targets. In addition, the CD4$^+$ T cells may mediate direct anti-viral effects through their production of IFN-γ and TNF-α. Cytokines may play an important role in protection against viruses such as VV and MCMV, as these viruses are known to be very sensitive to IFN-γ (Muller et al., 1994; Orange and Biron, 1996; Van Den Broek et al., 1995).

It has been known for many years that immunologically naive hosts respond poorly to antigens, but I believe that my observations reflect specificities in responses rather than simply an increased pool of memory T cells ready to be nonspecifically stimulated. I could not detect any enhanced cytokine production by memory T cells from LCMV-immune mice challenged with PV- or from VV-immune mice challenged with LCMV.
Thus, the frequency of cytokine-producing cells was dependent both on the virus-specific immune status of the animal as well as the particular challenge virus.

Cross-reactive T cell responses have now been described in several different systems ranging from in vitro work with T cell clones stimulated with altered peptide ligands to in vivo models showing cross-reactive T cells between two bacterial proteins or between unrelated viruses (Evavold et al., 1995; Harris et al., 1995; Selin et al., 1994). Additional work has shown that peptides with minimal primary sequence identity can elicit cross-reactive T cell responses (Hagerty and Allen, 1995; Wucherpfennig and Strominger, 1995), and in some cases alteration of the peptide can result in functionally different T cell responses, such as cytokine secretion and/or cytolytic responses in the absence of proliferation (Evavold and Allen, 1992; Evavold et al., 1993; Evavold et al., 1993; Hollsberg et al., 1995). Our laboratory’s demonstration by LDA of cross-reactive memory CD8+ CTL suggests that cross-reactive memory CD4+ T cells may also proliferate in some of these cultures (L.K. Selin and R.M. Welsh, unpublished observations). Interestingly, I have observed increased LCMV CD4+ Thp frequencies in PV+LCMV-immune mice as compared to LCMV-immune mice in four of six LDA. Taken together, these observations suggest that the production of cytokines elicited by heterologous viral infections may be due to cross-reactive memory CD4+ T cells. However, this has yet to be formally proven in LDA assays.
In experiments examining the fate of the LCMV-specific CD4+ T cells following multiple heterologous virus infections, I show that heterologous virus infections have little impact on the stable LCMV-specific CD4+ memory T cell pool. Thus, the memory CD4+ T cell frequency is resistant to reduction by heterologous virus infections, even under conditions that reduce the LCMV-specific CD8+ CTL frequency (Selin et al., 1996). Several reasons may account for why we observe a decline in the CD8+ CTL frequency but not in the CD4+ T cell frequency in this system. One possibility is that the CD4+ T cells are more cross-reactive between these viruses than the CD8+ CTL. The activation of virus-specific memory T cells via cross-reactivity seems to protect these cells from the deletion that occurs of the memory T cells specific to earlier viruses (L.K. Selin and R.M. Welsh, unpublished observations). The participation of memory CD4+ T cells to the increased IFN-γ production following VV infection of LCMV-immune mice would seem to support this notion (Fig. V-3). Moreover, I have noticed enhanced LCMV-specific CD4+ T cell frequencies in PV-immune mice, further suggesting that CD4+ T cells may be cross-reactive between some of these viruses. Another, more likely, possibility is that none of these virus infections induces as strong a CD4+ as a CD8+ T cell response. Even though, I have shown in Chapter IV that >10% of the CD4+ T cells score as virus-specific during infection with LCMV, a virus not thought of as a strong inducer of CD4+ T cells. The CD4 population does not expand in number like the CD8 population, which has to be substantially compressed after an infection. The LCMV-specific CD4+ T cell
frequency remained stable even after several protein antigen immunizations. One reason for this dichotomy between the CD8+ and CD4+ T cells may be the large difference in precursor frequencies generated following these virus infections (Selin et al., 1996; Varga and Welsh, 1998; Varga and Welsh, 1998). It may be that all of the available CD8+ T cell memory pool is filled after each infection, and therefore memory cells to earlier infections must be deleted to make room for the newly generated memory cells to the most recent infection. Since there are approximately 7- to 10-fold fewer CD4+ Thp than CD8+ CTLp (Murali-Krishna et al., 1998; Varga and Welsh, 1998), it may be that the CD4+ T cell memory pool has not become full following just 4 virus infections. Thus, there would be no need to delete any memory T cells from earlier infections. In addition, there is a large expansion in CD8+ T cell numbers following each of these virus infections, and once virus has been cleared, the immune system must return the T cell numbers back to homeostasis through activation induced cell death of the activated T cell population. During this contraction phase following a virus infection, memory T cells specific to previous viruses may become sensitized to die in this apoptotic environment.

These experiments also show that the CD4+ and CD8+ memory T cell pools are regulated independently. In experiments performed in collaboration with Dr. L.K. Selin, we show that the LCMV-specific CD4+ Thp remains stable in mice that reduce the LCMV-specific CD8+ CTLp following heterologous virus infections (Figs. V-4, V-5 and (Selin et al., 1996)). It is currently unclear what mechanism drives this deletion of
memory CD$^+$ T cells. However, these experiments demonstrate that whatever the mechanism is, it deletes the virus-specific CD$^+$ T cells without affecting the CD$^+$ T cells. This suggests that different signals may regulate the size of the CD$^+$ and CD$^+$ T cell memory pools.

The resistance of the LCMV-specific memory CD$^+$ T cell pool to deletion following heterologous virus infections may play a role in preserving the remaining virus-specific CD$^+$ CTLp. Protection against each of the viruses used here is dependent on MHC class I-restricted CD$^+$ CTL, which can function independently of CD$^+$ T cells to mount a protective response. However, recent work has suggested that CD$^+$ T cells may play a role in the maintenance of memory CD$^+$ CTL to viruses such as LCMV (Von Herrath et al., 1996). With data presented in this thesis, our laboratory has now shown that LCMV-specific memory CD$^+$ and CD$^+$ T cells persist long after the initial infection and that there is a continually cycling population of CD$^+$ CTLp (Razvi et al., 1995; Varga and Welsh, 1998). This suggests that the continued presence of memory CD$^+$ T cells may be required to maintain the continued cycling of a small frequency of memory CD$^+$ CTLp. Thus, the maintenance of the CD$^+$ Thp frequency may help to maintain the remaining virus-specific CD$^+$ CTLp to ensure long-term protective immunity. It is currently unknown whether or not the maintenance of the CD$^+$ T cell memory pool requires the presence of CD$^+$ T cells or some other cell population.
F. Exhaustion of virus-specific CD4+ T cells. Many of the above experiments clearly establish the stability of LCMV-specific CD4+ T cell memory. I have presented data showing that the LCMV-specific CD4+ Tp frequency remains stable even under conditions that result in the reduction of the LCMV-specific CD8+ CTLp frequency (Selin et al., 1996). Highly activated T cells are more susceptible than unstimulated cells to undergo apoptosis when they receive continued stimulation through their T cell receptors (Razvi and Welsh, 1993). Infection of mice with a high-dose of LCMV Clone 13 has been shown to selectively delete the virus-specific CD8+ T cells by days 7-8 p.i. due to the overwhelming virus infection (Moskophidis et al., 1993). The fate of the virus-specific CD4+ T cells under these conditions was not known. In this thesis I have provided evidence that there is a decline in the detectable frequency of IL-2-producing CD4+ Tp as detected using the CD4 LDA (Table VI-1). In addition, I show that a large percentage of the CD4+ T cells continue to express activation markers following LCMV Clone 13 infection and that some of these CD4+ T cells are still capable of making IFN-γ following stimulation with peptide in the presence of IL-2 (Table VI-2 and Fig. VI-2). These results suggest that the CD4+ T cells may become anergic following a high-dose LCMV Clone 13 infection and are thus impaired in their ability to produce IL-2 but can be rescued to make IFN-γ in the presence of peptide and exogenous IL-2. This interpretation would agree with a recent study demonstrating that LCMV-specific
transgenic CD4+ T cells persist in vivo in an anergic state for >6 weeks following high-dose LCMV Docile infection (Oxenius et al., 1998).

Recent work examining HIV-infected humans has correlated decreased CD4+ T cell responses with elevated viral loads (Rosenberg et al., 1997). Thus, the progressive loss of virus-specific CD8+ CTL activity that occurs during HIV infection seems to parallel the decline in the CD4+ T cell numbers. The underlying mechanisms by which CD4+ T cells maintain CD8+ CTL during persistent viral infections are not well understood. The CD8+ T cells may need cytokines, such as IL-2, secreted by the CD4+ T cells in order to survive and not become exhausted in the high antigen environment. Another possibility is that CD4+ T cells sustain the activation of professional APC. CD4+ T cells promote the activation of dendritic cells through the interaction of CD40-ligand, on the CD4+ T cell, and CD40, on the dendritic cell. Recent work has suggested that dendritic cells play a critical role in stimulating naive CD8+ T cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). Thus, during persistent virus infection, CD8+ T cells may need the CD4+ T cells to maintain the activation of the APC in order to continue the priming of new CD8+ CTL. This point is supported by the observation that mice genetically deficient in perforin do not clonally exhaust as the CD8+ T cells cannot destroy virus-infected APC (Binder et al., 1998; Gallimore et al., 1998). A recent report has shown that mice deficient in CD40-ligand fail to efficiently control LCMV after apparent resolution of the acute infection, as virus would recrudesce several months after
the initial infection (Thomsen et al., 1998). Interestingly, the data I present here suggest that the LCMV-specific CD4+ T cells functionally exhaust after the CD8+ T cells have exhausted (Moskophidis et al., 1993), indicating that even in the presence of CD4+ T cells, the CD8+ T cells may become activated so fully in this high antigen environment that they are driven into activation induced cell death.

G. Conclusions. The data presented in this thesis help provide a better understanding of the CD4+ T cell response during virus infections as well as highlight some important similarities and differences between the CD4+ and CD8+ T cell responses during acute viral infections. I have made several novel observations in the course of these studies including:

1. CZ-1, a mAb generated in our laboratory, represents a novel activation and memory marker for murine CD4+ T cells.

2. LCMV infection induces primarily a Th1 virus-specific cytokine response.

3. There is a more profound CD4+ T cell response during LCMV infection than has ever been previously recognized.
4. The LCMV-specific memory CD4$^+$ Tp remains extremely stable into long-term immunity. In addition, heterologous virus infections fail to significantly perturb this stable CD4$^+$ memory T cell pool, even under conditions that result in the reduction of CD8$^+$ memory T cells.

5. CD4$^+$ T cells can contribute to heterologous T cell-mediated immunity through the production of cytokines such as IFN-γ.

6. CD4$^+$ T cells become unresponsive following a high-dose LCMV infection.

The mechanisms that underlie some of these important observations remain to be identified. However, this thesis provides a foundation for further studies examining the role of CD4$^+$ T cells during viral infections.
CHAPTER VIII

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