The Role of CD4⁺ T cells in the CD8⁺ T cell Response to Vaccinia Viral Infection

by

Patricia L. Novy

Department of Immunology
Duke University

Date:_______________________
Approved:

___________________________
Yiping Yang, Supervisor

___________________________
Yuan Zhuang, Chair

___________________________
Garnett Kelsoe

___________________________
Thomas Kepler

___________________________
Weiguo Zhang

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2010
ABSTRACT

The Role of CD4+ T cells in the CD8+ T cell Response to Vaccinia Viral Infection

by

Patricia L. Novy

Department of Immunology
Duke University

Date:_______________________

Approved:

___________________________

Yiping Yang, Supervisor

___________________________

Yuan Zhuang, Chair

___________________________

Garnett Kelsoe

___________________________

Thomas Kepler

___________________________

Weiguo Zhang

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Immunology in the Graduate School of Duke University

2010
Copyright by
Patricia L. Novy
2010
Abstract

The role of CD4 T cell help in primary and secondary CD8 T cell responses to infectious pathogens remains incompletely defined. The primary CD8 T response to infections was initially thought to be largely independent of CD4 T cells, but it is not clear why some primary, pathogen-specific CD8 T cell responses are CD4 T cell-dependent. Furthermore, although the generation of functional memory CD8 T cells is CD4 T cell help-dependent, it remains controversial when the “help” is needed. The goal of this thesis project is to determine requirement and mechanisms of CD4 help during the CD8 response to vaccinia viral (VV) infection.

The first aim of this project was to determine when CD4 T cell help is required during the CD8 response to VV infection. Using both CD4-deficient mice and mice with transient depletion of CD4 T cells, we demonstrated that CD4 T cell help was not needed for the activation and effector differentiation of CD8 T cells during the primary response to VV infection. However, the activated CD8 T cells showed poor survival without CD4 T cell help, leading to a reduction in clonal expansion and a diminished, but stable CD8 memory pool. In addition, we observed that CD4 T cell help provided during both the primary and secondary responses was required for the survival of memory CD8 T cells during recall expansion. Our study indicates that CD4 T cells play a crucial role in
multiple stages of CD8 T cell response to VV infection and may help to design effective vaccine strategies.

Given that CD4 T cell help is critical for the survival of activated CD8 T cells during both the primary and memory recall responses, it is still unclear how CD4 T cell help promotes CD8 T cell survival. The second aim of this project was to determine the mechanism of CD4 help for the survival of activated CD8 cells. We first showed that CD4 help in vitro was mediated by IL-21, a cytokine produced predominantly by activated CD4 T cells. We then demonstrated direct action of IL-21 on CD8 T cells was critical for the VV-specific CD8 T cell response in vivo. This intrinsic IL-21 signaling was essential for the survival of activated CD8 T cells and the generation of long-lived memory cells. We further revealed that IL-21 promoted CD8 T cell survival in a mechanism dependent on activation of the STAT1 and STAT3 pathways and subsequent upregulation of the pro-survival molecules Bcl-2 and Bcl-xL. Collectively, these results identify a critical role for CD4-derived IL-21 signaling in CD8 T cell responses to acute VV infection in vivo and may help design effective vaccine strategies in situations where CD4 cells are not fully functional.
Dedication

This thesis is dedicated to the memory of my grandmother, Betty Galvan. She taught me, rather fearlessly, that nothing worth doing in life should be done halfway. I could not have done any of this work without her lasting guidance and influence.
Contents

Abstract .........................................................................................................................................iv
List of Tables ................................................................................................................................... xi
List of Figures .................................................................................................................................. xii
List of Abbreviations .................................................................................................................... xiii
Acknowledgements ........................................................................................................................xvi
1. Introduction ............................................................................................................................... 1
  1.1 The CD8 T cell response to acute infection ........................................................................ 1
  1.1.1 Expansion and formation of the effector pool ......................................................... 2
  1.1.2 Contraction .................................................................................................................... 4
  1.1.2 Stabilization and memory formation ............................................................................ 5
  1.2 Characteristics of memory CD8 T cells .......................................................................... 5
  1.2.1 Memory cell subsets ..................................................................................................... 7
  1.3 Factors governing the CD8 T cell response .................................................................... 9
  1.3.1 Role of the T cell receptor and costimulation .............................................................. 9
  1.3.2 CD4 T cell help ........................................................................................................... 10
  1.3.2.1 CD4 T cells ............................................................................................................ 10
  1.3.2.2 Role of CD4+ T cells during the primary CD8 response ...................................... 12
  1.3.2.3 Role of CD4 help during the CD8 recall response ............................................... 14
  1.4 Models to study the CD8 T cell response to vaccinia viral infection .............................. 16
  1.4.1 Tracking the response to VV infection using transgenic CD8 T cells ................. 17
1.4.2 Tracking the endogenous CD8 T cell response to VV infection .................. 18
1.5 Thesis Prospectus .................................................................................................................. 19
2. Materials and Methods ........................................................................................................... 21
2.1 Mice ........................................................................................................................................ 21
2.2 Adoptive transfer of T cells .................................................................................................. 22
2.3 Immunizations and antibody treatment .............................................................................. 22
2.4 Antibodies and flow cytometry ........................................................................................... 23
2.5 Intracellular staining .............................................................................................................. 24
2.5.1 Intracellular cytokine staining ...................................................................................... 24
2.5.2 Intracellular transcription factor staining ........................................................................ 24
2.6 Cell Isolation .......................................................................................................................... 25
2.6.1 Memory CD8 T cell isolation ....................................................................................... 25
2.6.2 Isolation of lymphocytes from non-lymphoid tissues .................................................... 25
2.6.3 Activated DC isolation .................................................................................................... 25
2.7 Stimulation of T cells in vitro ............................................................................................ 26
2.7.1 DC-mediated stimulation ............................................................................................... 26
2.7.1 Antibody-mediated stimulation ..................................................................................... 26
2.8 Real-time quantitative PCR ................................................................................................... 27
2.9 Ovary vaccinia virus titer assay .......................................................................................... 27
2.10 Retrovirus preparation and infection ................................................................................. 28
2.11 Statistical Analysis .............................................................................................................. 28
3. CD4 T cells are required for CD8 T cell survival during both primary and memory recall responses .......................................................... 29

3.1 Introduction ........................................................................................................................... 29

3.2 Results........................................................................................................................ .............32

3.2.1 CD4 T cell help is needed for clonal expansion of CD8 T cells during the primary response to VV infection .......................................................... 32

3.2.2 CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help .......................................................... 35

3.2.3 The survival of activated CD8 T cells during priming is dependent on CD4 T cell help .................................................................................................................. 39

3.2.4 Diminished, but relatively stable CD8 memory pool can develop in absence of CD4 T cells .................................................................................................................. 41

3.2.5 CD4 T cell help is also required for CD8 memory recall expansion following secondary challenge ....................................................................................... 45

3.2.6 CD4 T cell help promotes the survival of CD8 T cells during recall expansion ......49

3.3 Discussion ..............................................................................................................................51

4. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection .......................................................... 57

4.1 Introduction ........................................................................................................................... 57

4.2 Results........................................................................................................................................ 60

4.2.1 CD4 T cell help for CD8 T cell survival is mediated by IL-21 .............................................. 60

4.2.2 The priming of VV-specific CD8 T cells in vivo is dependent on intrinsic IL-21 signaling ......................................................................................................................... 66

4.2.3 Intrinsic IL-21 signaling is critical for the survival of activated CD8 T cells in vivo 68

4.2.4 Intrinsic IL-21 signaling is required for the formation of memory CD8 T cells in vivo ............................................................................................................................... 73
4.2.5 IL-21 enhances the survival of activated CD8 T cells via the STAT1 and STAT3 pathways......................................................................................................................................................76

4.2.6 IL-21 signaling on CD8 T cells promotes activation of STAT1 and STAT3 and induction of Bcl-xL in response to VV infection in vivo..........................................................80

4.3 Discussion ..................................................................................................................................................................................82

5. Overall Conclusions and Future Directions .........................................................................................................................86

References .......................................................................................................................................................................................95

Biography .....................................................................................................................................................................................107
List of Tables

Table 1: rVV-HA titer in ovaries at days 3 and 28 postinfection........................................42
List of Figures

Figure 1: Defective clonal expansion of CD8 T cells in the absence of CD4 T cell help during a primary response to VV infection ................................................................. 34

Figure 2: CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help. ............................................................................. 37

Figure 3: CD4 T cells promote the survival of activated CD8 T cells during priming in vivo .......................................................................................................................... 40

Figure 4: Diminished, but relatively stable CD8 memory pool can develop in the absence of CD4 T cells .......................................................................................................................... 43

Figure 5: Phenotypic and function analyses of the “helpless” memory CD8 T cells ...... 44

Figure 6: CD4 T cell help provided both during initial priming and following rechallenge is required for recall expansion of memory CD8 T cells ......................... 47

Figure 7: The survival of memory CD8 T cells during a recall expansion is dependent on CD4 T cells. .......................................................................................................................... 50

Figure 8: CD4 T cell help for CD8 T cell survival is mediated by a soluble factor in vitro ................................................................................................................................. 63

Figure 9: CD4 T cell help for the survival of activated CD8 T cells in vitro is mediated by IL-21 .......................................................................................................................... 64

Figure 10: Intrinsic IL-21 signaling is required for the priming of VV-specific CD8 T cells in vivo ............................................................................................................................. 67

Figure 11: CD8 T cell clonal expansion in response to VV infection is compromised in the absence of IL-21 signaling ................................................................................................. 70

Figure 12: IL-21 signaling is required for the survival, but not the activation or proliferation, of CD8 T cells .................................................................................................................. 72

Figure 13: Defective CD8 memory formation in the absence of IL-21 signaling .......... 74

Figure 14: IL-21 activates the STAT1 and STAT3 signaling pathway, leading to the enhanced survival of CD8 T cells in vitro. ......................................................................................... 78
Figure 15: IL-21 signaling promotes activation of the STAT1 and STAT3 pathways and upregulation of Bcl-xL in vivo.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Ad-HA</td>
<td>Recombinant adenovirus expressing hemagglutinin</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD4-deficient</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus 1</td>
</tr>
<tr>
<td>ICS</td>
<td>Intracellular cytokine staining</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for activation of T cells</td>
</tr>
<tr>
<td>Lck</td>
<td>Leukocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph nodes</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MSCV</td>
<td>Murine stem cell virus</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming units</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PLN</td>
<td>Peripheral lymph nodes</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>rVV-HA</td>
<td>Recombinant vaccinia virus expressing hemagglutinin</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>STAT3-D</td>
<td>Dominant-negative STAT3</td>
</tr>
<tr>
<td>Tcm</td>
<td>Central memory T cells</td>
</tr>
<tr>
<td>Tem</td>
<td>Effector memory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia virus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
Acknowledgements

Much like Hillary Clinton pointed out with raising children, getting through graduate school takes a village. I have had measureless help and support during the last five years, without which I could not have accomplished the work in this thesis.

First and foremost, I would thank my mentor, Dr. Yang. Dr. Yang took a chance on a naïve and still relatively unaware student and has spent countless hours devoted to teaching me about science and, more importantly, how to think about science. I am incredibly grateful for his time and profound patience while I learned about techniques and began to create my own ideas. His vast expertise and wide topical knowledge have taught me the importance of innovative thinking and looking at things on a much larger scale. He has undoubtedly shaped who I am as a scientist.

I would also like to thank my committee members Dr. Yuan Zhuang, Dr. Garnett Kelsoe, Dr. Weiguo Zhang, and Dr. Tom Kepler. They have had incredible insight into remaining questions and how to address the technical issues I have had. I have been very fortunate to be able to approach them any time and ask them anything from how to fix my culture systems to what I am going to do with my Ph.D. someday. I am extremely grateful for their advice and direction.

Working in the Yang lab has been a testament to the incredibly diverse personalities of scientists. I would first like to thank Michael Quigley, who was the
acting big brother of the lab and who taught me nearly every technique I know. I would also like to sincerely thank Ana Sanchez and Jennifer Martinez, without whom the lab would be an incredibly dull place. In addition, I thank past and present members of the Yang lab, Jiangao Zhu, Yuqing Yuan, Chunbo Wang, Ian Horkheimer, Yufeng Zhou, Xiaopei Huang, Joshua Brandstadter, Kotaro Yoshida, Jonathan Henzel, Todd Brennan, Liwen Li, and Jessica Mangum, for their encouragement, ideas, and support.

I am incredibly lucky to have a wonderful family who has encouraged me throughout my time at Duke. First, I have to thank my mother, Judith “the Judes” Novy. She is the first person to get the call when things fail or when things go unexpectedly well. I am so grateful for her ability to listen, to come up with fabulous ways to celebrate success, and to diffuse that horrible feeling that everything is lost. She is my rock. Next I have to give my overwhelming thanks to my father, Bob Novy, who has been my biggest fan. He has taught me to keep my head up and my eye on the prize. I never would have made it through graduate school without his support. I also want to thank my brother Christopher. In addition to his highly entertaining life stories, he has constantly shown me how lucky I am to be able to go to school and how valuable this education really is. I would also like to thank Paula Proctor, Dennis Galvan, Susan Sanders, Greg Galvan, Michael Immel, and Ellen and Beth Robinson for their love and support.
In 2005, I came to Durham and left my family in California. Now 5 years later, I have my own North Carolina family. The first members of this family were the members of my class (the first all-female class) and I am eternally grateful to Mariana Chuck, Tamara Lundeen, Jennifer Martinez, Pilar Snowden, and Ashley St. John for their lifelong friendship. This family now also includes my two of my greatest friends, Deirdre Fuller and Lisa Weir. I am so lucky to have people who can give me advice and who are my biggest supporters. I also would like to thank John Garnett, who has been a constant source of encouragement and, when it was necessary, distraction from the sometimes overwhelming task of working toward a Ph.D.

I would also like to thank the entire Department of Immunology. The faculty, post-doctoral fellows, graduate students, and technicians have provided a fabulous and cooperative working environment. The Zhang, Kelsoe, Tedder, Krangel, and Zhuang labs have been extremely generous with their time and reagents and for that I am very thankful. I would also like to thank Hrisavgi Kondilis-Mangum, Shudan Shen, Beth Jones, Derek Cain, Matt Holl, David Dilillo, Eva Chung, Tommy O’Brien, Han-yu Shih Elizabeth Chan, and Claudia Ou-Yang for their expertise in various techniques as well as thoughtful advice on planned experiments.
1. Introduction

1.1 The CD8 T cell response to acute infection

A key part of the adaptive arm of the immune system is the CD8 T cell response. CD8 T cells contribute to host defense during both acute and chronic infections with viruses and intracellular bacteria, as well as participate in the elimination of tumor cells. Because of their critical role as the killer cells of the adaptive immune system, there is substantial interest in understanding the pathways involved in the generation and regulation of the CD8 T cell response and the soluble or cell-associated signaling pathways that influence the generation of effector and memory cells during both acute and chronic infections [1].

CD8 T cell development occurs in the thymus, where the developing cells are positively selected on the basis of the affinity threshold of their T cell receptors (TCRs) for self-peptides in the context of major histocompatibility complex (MHC) molecules expressed on thymic epithelial cells. After this positive selection, cells that bear high affinity TCRs for self-peptides are removed in a process termed negative selection [2]. The remaining cells exit to the periphery where they form a heterogeneous pool of naïve cells. This pool is made up of T cells with a variety of TCRs that are able to respond to a vast array of different peptide antigens. The precursor frequency for cells that can respond to a specific peptide antigen are very low; studies in mice estimate that there are
approximately 15-1000 antigen-specific CD8 T cells for any given MHC/peptide combination [3, 4].

The naïve T cell pool persists in the periphery, waiting for encounter with their cognate antigen. Homeostasis of these naïve T cells is achieved through a combination of cytokine and low-grade TCR signaling; interaction with MHC molecules in the periphery is absolutely essential for the survival of these cells [5, 6]. Naïve T cell survival is also dependent on signaling through the interleukin (IL)-7 receptor (CD122) on naïve T cells [7]. IL-7 mediates the proliferation and survival of naïve T cells by regulating cellular metabolism and glucose uptake in a PI3 kinase-dependent manner [8]. The bioavailability of IL-7 serves to regulate the size of the naïve T cell pool, keeping it relatively stable until the naïve cells are needed to combat infection [9].

1.1.1 Expansion and formation of the effector pool

During the adaptive immune response to acute infection, naïve CD8 T cells expand and form an effector pool capable of clearing the invading pathogen. The initiation of the CD8 T cell response occurs in a highly orchestrated process by which the antigen, an 8-11 residue peptide in the context of an MHC Class I molecule, is enriched in the T cell zones of lymphoid organs on antigen-presenting cells (APCs) [10]. These APCs present both endogenous antigens and foreign antigens that have gained access to the cytosol of the APC for processing by the MHC class I presentation pathway.
Additionally, dendritic cells (DC) are able to “cross-present” antigen, a process by which antigens obtained from apoptotic or infected cells are presented on MHC class I to naïve CD8 T cells [11, 12]. After interaction of the TCR with its cognate antigen, the CD8 T cell becomes activated and undergoes several rounds of proliferation. In this manner, what was once a virtually undetectable pool of antigen-specific cells can comprise as much as 1-2% of splenic CD8 T cells in certain bacterial infections or as much as 50% of splenic CD8 T cells in some viral infections [13-15].

In addition to this robust expansion, these cells acquire several effector functions to combat the infection, such as expression of the proinflammatory cytokines IFN-γ and TNF-α and production of the cytolytic molecules perforin and granzyme. They also gain the ability to migrate to virtually all tissues of the body [16]. This differential migratory ability is a result of changes in the expression of several cell surface molecules. CD44, which binds to hyaluronic acid and facilitates homing to peripheral tissues, becomes upregulated after T cell activation [17]. CD62L, also known as L-selectin, which mediates homing to lymph nodes through high endothelial venules, and CC chemokine receptor 7 (CCR7), a chemokine receptor that is crucial for homing to lymph nodes, are downregulated to allow the cells to migrate to peripheral tissues [17]. Combined, these surface marker changes result in the differential migratory pattern of effector CD8 T cells away from the secondary lymphoid organs and into the site of infection.
Upon activation, effector CD8 T cells become less dependent on IL-7 signaling. To this end, the expression of the IL-7 receptor (CD127) is reduced in effector CD8 T cells. This reduction in expression is only transient though; CD127 expression is restored in the cells that will go on to form memory cells [18, 19]. These changes in cell surface molecule expression allow the effector pool to be phenotypically defined by the expression of high levels of CD44, and low levels of CD62L, CCR7, and CD127 [10, 20].

1.1.2 Contraction

After this rapid expansion phase and the resolution of infection, the effector cell population undergoes a dramatic contraction phase, where approximately 90 to 95% of cells undergo programmed apoptosis. This catastrophic cell death is mediated largely by the ratio of expression of survival factors, such as Bcl-2 and Bcl-xl, versus apoptotic factors, such Bim and Fas [21-25]. Survival of antigen-specific cells is dependent on IL-7 signaling; absence of IL-7 signaling leads to reduced cell numbers surviving the contraction phase [26]. IL-7 promotes cell survival by increasing levels of Bcl-2, which is necessary to protect cells from Bim-mediated cell death [25].

Accumulating evidence supports a role of inflammation as a key regulator of contraction. Original studies using antibiotics to diminish *Listeria* infection demonstrated that the reduction of inflammatory factors severely blunted the extent of contraction of the CD8 effector pool [27, 28]. Further studies showed that the addition of
adjuvant, namely CpG, to peptide-pulsed DC immunizations enhanced both the expansion and contraction of antigen-specific CD8 T cells [29, 30]. Collectively, these studies have pointed to the role of inflammatory cytokines, namely IFN-γ and IL-12, as key regulators of the extent of contraction: higher levels of secreted IFN-γ and IL-12 lead to higher degrees of CD8 T cell expansion and subsequent contraction [27, 28, 31-33]. Expression of the IL-7 receptor alpha chain at the peak of infection is largely thought to mark the effector cells that will survive this contraction phase and become memory cells [19].

1.1.2 Stabilization and memory formation

While most of the effector CD8 T cells die during the contraction phase, the remaining few persist into the stabilization phase. At this point, these surviving cells differentiate to form the CD8 T cell memory pool. This pool is numerically larger than the original naïve pool and thought to be stable for the lifetime of the host [34]. The entire antigen-specific CD8 T cell response results in a memory pool that is phenotypically and functionally different than its naïve or effector precursors [35].

1.2 Characteristics of memory CD8 T cells

Immunological memory is commonly defined as the faster and stronger response of the immune system that follows secondary exposure to the same antigen. Therefore, CD8 memory is typically defined by its functional relevance: the ability to rapidly re-
differentiate into effectors should the cells encounter their cognate antigen again. After this restimulation, memory cells are much faster than naïve cells at entering cell cycle, synthesizing cytokines, differentiating into cytolytic effectors, and migrating into non-lymphoid tissues [36-41]. The mechanisms underlying this rapid recall ability of memory CD8 T cells are still largely unclear. Several studies have shown that TCR signaling in memory cells has a much lower threshold. One study attributed this to elevated resting levels of phosphorylated LAT, which is the active form of a TCR-proximal scaffolding molecule, leading to the more efficient accumulation of phosphorylated downstream signaling molecules in memory CD8 T cells [42]. Furthermore, the TCR proximal kinase Lck has been found to be dispensable for the recall response of memory T cells [43, 44]. In addition to the rapid-relay of TCR signals in memory cells, several lines of evidence also support the idea that resting memory CD8 cells are in a “ready-to-respond” state due to distinct chromatin modifications and mRNA expression profiles different from their naïve counterparts [45]. The underlying mechanisms determining these differences are still under investigation.

Another important characteristic of memory cells is their persistence. The homeostasis of the memory T cell pool is mediated by a dynamic process that involves balancing a low-level of cytokine-mediated proliferation with cell death, a process
known as memory turnover [7, 9, 46, 47]. This turnover does not require antigen or the interaction of the TCR with MHC Class I molecules [6, 48, 49].

Clear roles for IL-15 and IL-7 in this cytokine-mediated turnover have been described. Three different groups have shown that IL-15 is involved in the maintenance of antigen-specific memory CD8 T cells and the absence of the highly expressed IL-15 receptor leads to a reduction in the numbers of memory cells [9, 46, 50]. IL-15 functions by promoting the division of memory CD8 T cells to maintain their numbers in the periphery [46, 50]. Although necessary for homeostatic proliferation, IL-15 is not essential for memory cell function [46]. Since memory CD8 cells also express high levels of the IL-7 receptor, studies elucidating the role of IL-7 signaling in memory CD8 T cells have demonstrated that IL-7 is responsible for the survival of a population of memory cells [51]. Specifically, IL-7 may support the survival of a subset of memory cells that do not constitutively divide in response to IL-15, such as that seen in the absence of the IL-15 receptor [46, 50]. Together, IL-7 and IL-15 cooperate to maintain the CD8 memory pool; IL-15 maintains cell turnover, while IL-7 promotes survival.

1.2.1 Memory cell subsets

While CD8 memory T cells are classically defined by their functionality, they consist of a heterogeneous population with respect to surface molecules, organ homing, and effector molecules. Traditionally, memory cells are divided into two subsets based
on the expression of different homing molecules, specifically CD62L and CCR7 [16, 52].

Central memory T cells (Tcm) are defined as CD62L$^{\text{high}}$CCR7$^{-}$ and reside in secondary lymphoid tissues [53]. Conversely, effector memory cells (Tem) are defined as CD62L$^{\text{low}}$CCR7$^{-}$ and reside in nonlymphoid tissues such as the liver, lung, and intestine [10, 53]. In addition to their different migratory potentials, they also exhibit different functional abilities. Tcm are not actively cytolytic but have a higher proliferative capacity upon restimulation than Tem, which show constitutive direct cytolytic activity [10, 53].

The relationship between central and effector memory cells is still unclear. While early work suggested that Tcm is generated from Tem, more recent studies indicate that Tem do not convert to Tcm although the opposite can occur [54, 55]. This unclarity may be due to an incomplete definition of the cell surface phenotypes of the cells of these two populations; the strict classification of memory cells based on the expression of CD62L and CCR7 is likely inconclusive since studies have demonstrated variability within these subsets as well as changes in individual surface phenotypes within these subsets [56, 57]. Furthermore, differences in infectious agent, route of infection, and persistence of infection all have profound effects on the phenotype, functionality, and survival of the different memory subsets [58]. Therefore, studies examining the differentiation and maintenance of these subsets must be taken in context of the experimental design.
1.3 Factors governing the CD8 T cell response

A goal of vaccine development is to understand how different factors affect the CD8 T cell response and how they can be subsequently modified to achieve an optimal response. To this end, it is crucial to define the parameters that regulate the development, maintenance, and functionality of the CD8 T cell response to acute infection.

1.3.1 Role of the T cell receptor and costimulation

The TCR is comprised of the αβ TCR chains and the CD3 chains. Upon the binding of the TCR to cognate peptide-MHC complexes presented by APCs, several residues in the CD3 coreceptor chain are phosphorylated which leads to the phosphorylation of LAT and subsequent recruitment and activation of ZAP-70 and the Src-family kinases Lck and Fyn, effectively initiating downstream signaling pathways that lead to proliferation and differentiation [59, 60]. This TCR engagement, commonly referred to as signal 1, lasts approximately 24 hours and enables CD8 T cells to commit to a program of division and differentiation without any further stimulus to become effector and memory cells [61-63]. Costimulatory signals, also referred to as signal 2, compound with TCR signals and prevent the anergy or clonal deletion that can occur when cells receive TCR signals alone [64]. This occurs through engagement of CD28 on T cells with one of its ligands, CD80 or CD86, present on mature APCs [65, 66]. In
addition to signal 1 and signal 2, there is an emerging body of work suggesting that a signal 3, mediated by the inflammatory cytokines IL-12 and type I IFNs, is crucial for driving T cell expansion, function, and survival [67-70].

1.3.2 CD4 T cell help

Although it has been clearly demonstrated that signals 1, 2, and 3 are sufficient to induce the CD8 T cell response, the role of CD4 T cell help is still under investigation. Several groups have demonstrated that CD4 T cells are required at some phase of the CD8 immune response for optimal pathogen clearance and protection from reinfection, but the exact timing and mechanisms of this help are still unclear [1, 35, 71, 72].

1.3.2.1 CD4 T cells

CD4 T cells, like CD8 T cells, develop in the thymus. They undergo both positive and negative selection to generate a pool of naïve T cells that are capable of interacting with self-MHC but are not strongly self-reactive. The CD4 T cell recognizes its cognate antigen in the context of MHC class II molecules. Upon TCR recognition and costimulation the naïve CD4 T cell will undergo a process of proliferation and differentiation to generate a pool of effector CD4 T cells, which are responsible for connecting antigen presentation by APCs to the functional activity of the immune response. Classically, this differentiation generates either Th1 cells, which promote cellular immunity and host protection to intracellular pathogens by helping the CD8 T
cell response, or TH2 cells, which secrete IL-4 and promote humoral immunity and protection to extracellular pathogens and parasites [73, 74]. Recently, a novel lineage termed TH17 has been described. These cells produce high levels of the proinflammatory cytokines IL-17, IL-6, and TNF that contribute to the pathology of a variety of inflammatory models such as contact hypersensitivity, experimental autoimmune encephalitis, psoriasis, and collagen-induced arthritis [75-78]. The outcome of the differentiation process relies heavily on the microenvironment of the naïve CD4 cell undergoing stimulation. If a naïve CD4 T cell is primed in the presence of IL-12, the cell enters into the TH1 differentiation program and expresses the transcription factor T-bet, which both induces production of IFN-γ and suppresses the expression of TH2 cytokines [79, 80]. Conversely, if the naïve cell is primed in the presence of IL-4, it will upregulate expression of GATA3, a regulator that is both necessary and sufficient for TH2 lineage differentiation [79, 81]. Finally, if the naïve cell is primed in the presence of IL-6 and TGF-β, it will upregulate RORγt which induces transcription of IL-17 and induces commitment to the TH17 lineage [82-85]. The master-regulators of this whole process are the activated innate antigen-presenting cells. They are responsible for secreting the cytokines that skew the lineage commitment of CD4 T cells to generate an appropriate immune response for a specific pathogen [86].
1.3.2.2 Role of CD4+ T cells during the primary CD8 response

While it is well-established that TCR recognition of MHCI-peptide in conjunction with costimulatory signals results in activation of a naïve T cell, the roles of cell-cell interactions influencing the CD8 response are still unclear. The role of CD4 T cell help for priming the CD8 T cell response was first described for non-inflammatory antigens, such as male minor HY antigen and MHC mismatches that led to skin graft rejection [87, 88]. The original model for this CD4 help suggested that in the absence of inflammation, CD4 T cells were required to fully activate APCs to efficiently prime CD8 T cell responses [89]. It has been effectively demonstrated in models of soluble peptide, tumor antigen, and antigen-pulsed DC immunizations that CD40L on CD4 T cells interacts with CD40 on APCs, thereby “licensing” them, primarily by enhancing expression of CD80 and CD86 as well as inflammatory cytokine production, to fully stimulate the CD8 T cell response [90-95]. In contrast, CD4 T cell help during the response to infectious pathogens was largely thought to be irrelevant because high levels of inflammation and/or TLR stimulation may be able to facilitate complete activation of the APC, allowing it to sufficiently prime a CD8 response [72, 96, 97]. The primary response to acute LCMV infection, for example, has been shown to be independent of CD4 T cell help [98, 99]. In these studies, CD8 T cells primed in the absence of CD4 help were efficiently able to proliferate, clear the infection, and form memory cells with kinetics
similar to CD4-sufficient counterparts, indicating that CD4 help is dispensable for the primary CD8 response to LCMV.

Although some infectious model systems demonstrate that CD4 help is dispensable, several reports show that the primary response to certain pathogens, such as adenovirus, influenza virus, HSV-1, and *Listeria monocytogenes*, is dependent on CD4 T cell help [100-103]. Since direct CD40-CD40L interactions between CD4 and CD8 T cells are not involved in these infections, it is still unclear what role CD4 T cells play in these primary infections [104, 105].

In addition to DC licensing, activated CD4 T cells may secrete cytokines to promote the CD8 response. IL-2 was initially characterized as a potent T cell growth factor *in vitro* and is produced in large concentrations by activated CD4 T cells [106]. It was proposed that paracrine secretion of IL-2 by CD4 T cells may be important during CD8 T cell activation, but *in vivo* studies have demonstrated that IL-2 is dispensable during the primary expansion of CD8 T cells [87, 107, 108]. Studies focusing on the secretion of chemokines by activated CD4 T cells have determined a role for CCL3, CCL4, CXCL9, and CXCL10 in recruiting CD8 T cell to sites of infection or to activated APCs [91, 109, 110].

In addition to IL-2 and a variety of chemokines, activated CD4+ T cells can also produce high levels of IL-21 [111, 112]. IL-21 is a member of the common γ-chain
cytokine family, is produced by CD4 T cells and NKT cells, and its receptor is expressed on both resting and activated B, T, NK and dendritic cells [111, 113]. Addition of IL-21 to various culture systems yields higher numbers of cytolytic effectors, suggesting a role for IL-21 in expansion of activated CD8 T cells [114-116]. Recent studies using a model of LCMV infection found no role for IL-21 in the CD8 response to acute infection, but an absolute requirement for IL-21 signaling in CD8 T cells to clear a persistent viral infection [117-119]. Although these studies highlight a role for IL-21 signaling to maintain the effector function of CD8 T cells during a chronic viral infection, the primary CD8 responses have been shown to be independent of CD4 T cell help in the acute model of LCMV infection. Studies elucidating the role of IL-21 during acute infections that require CD4 help have not yet been published.

1.3.2.3 Role of CD4 help during the CD8 recall response

In addition to differential reports on the role of CD4 T cells during the primary response, there is an emerging body of work describing the role of CD4 T cells in generating functional CD8 memory. Several reports suggest that CD4 T cell help during the primary response is crucial to generate functional memory CD8 T cells. In these studies, although the primary response to antigens such as LCMV were independent of CD4 help, CD8 memory cells generated in the absence of CD4 T cells did not have the ability to respond to secondary antigenic challenge [98, 120, 121]. Whether this CD4 help
is “instructive” during priming or required for efficient homeostasis of the memory pool is still unclear [120, 122, 123]. In a model of LCMV infection, unhelped CD8 T cells underwent programmed apoptosis mediated by the TNF-related apoptosis-inducing ligand (TRAIL) during a secondary response [124, 125]. Another mechanism for memory programming is mediated by IL-2. Data utilizing IL-2 receptor-mutant mice have shown that although the primary response to infection is independent of IL-2 signaling, IL-2 is required during the primary response to generate memory cells capable of mounting robust secondary responses [123]. These data indicate a programming role for CD4 T cell help in producing functional CD8 T cell memory. It is still unclear whether CD4 T cells are physically required during the recall response for optimal CD8 T cell function. Unlike the studies in mice using LCMV infection, studies using HCV infection have determined that CD4 T cells are required to be present and activated during secondary challenge, even when virus-specific CD8 T cells were originally primed in the presence of CD4 help [126]. Conversely, other studies have suggested that the role of CD4 T cells is to maintain memory CD8 T cell numbers, perhaps by providing signals to accessory cells to produce the prosurvival cytokines IL-7 and IL-15 [72]. In these experiments, memory CD8 T cells were transferred from mice devoid of CD4 T cells into hosts with a normal CD4 T cell compartment, allowing the CD8 memory numbers to be maintained
over time [99]. Which mechanism of CD4 help is crucial during different pathogenic challenges is still under investigation.

Taken together, the vast array of data indicates that the requirement for CD4 T cell help may be pathogen-specific and/or depend on the route of infection [100, 101, 127]. One group determined that CD4 help was dispensable when antigen was delivered intradermally, whereas CD4 help was required for the intraperitoneal route of immunization [127]. Thus, more conclusive studies regarding the role and mechanisms of CD4 help need to be performed for each potential vaccine and therapeutic as they may differ for each.

1.4 Models to study the CD8 T cell response to vaccinia viral infection

As the response to acute infection seems to vary depending on the infectious agent studied, it becomes increasingly more crucial to study pathogens that are medically relevant. Vaccinia virus (VV) is a large double-stranded DNA virus of the poxvirus family. Other members of the poxvirus family include variola virus, which causes smallpox, monkeypox virus, camelpox virus, ectromelia virus, and cowpox virus [128]. Since these viruses are morphologically indistinguishable and antigenically similar, infection with one virus provides protection against the other family member viruses [128]. For this reason, VV was used to successfully eradicate smallpox worldwide.
Vaccinia virus enters cells by a process involving direct fusion of the virus envelope and the plasma membrane of the cell [129]. Because of this phenomenon, vaccinia is capable of infecting a variety of cell types [129]. Upon infection with vaccinia virus, cells will present viral antigens on MHC Class I molecules; if the infected cells is an activated APC, it is capable of stimulating a CD8 T cell response [130]. More efficient at generating a CD8 response, however, is the cross-presentation of antigens from infected cells by APCs [131]. In this pathway, APCs phagocytose apoptotic/necrotic infected cells and present these exogenous antigens on MHC Class I in a TAP-dependent process [132]. In this way, APCs can travel to lymphoid organs and initiate a CD8 response from an infection anywhere in the body.

Due to its exceptional use in vaccination, VV is the most studied of the poxvirus family. Currently VV has been developed as an expression vector for foreign genes and as a live recombinant vaccine for both infectious diseases and cancer [133-135]. Since it is an exceptional vaccination tool, understanding the immunologic processes of VV clearance and protection are critical to designing the optimal vaccination scheme for a given ailment.

1.4.1 Tracking the response to VV infection using transgenic CD8 T cells

An important tool for tracking the CD8 T cell response is the TCR transgenic mouse, in which every CD8 T cell has a TCR with the same specificity. This transgenic
system allows for more detailed phenotyping and analysis of a specific cell population, generating significant insight into cellular events after priming, during contraction, and throughout memory response. It also allows the comparison of wild-type and gene-deficient CD8 T cells responding to infection as well as the effects of altered cellular environments on the CD8 response when gene-deficient hosts are used as recipients.

As laboratory techniques have enabled the manipulation of VV to express a variety of different exogenous antigens, a vast number of different recombinant VV strains have been developed [134]. One such recombinant strain encodes for the expression of influenza hemagglutinin (HA). With this strain, rVV-HA, it is possible to track an antigen-specific CD8 T cell response using CD8 T cells from a TCR transgenic mouse that recognize a K\(^d\)-restricted HA epitope as well as express the congenic marker Thy1.1. These cells can be transferred into congenic mice (Thy1.2\(^+\)) and tracked by the expression of Thy1.1 in vivo. Using this system, the CD8 T cell response to rVV-HA has been characterized [136]. The efficacy of this system allows low numbers of HA-specific CD8 T cells to be transferred, thereby reducing the possibility that artificially high numbers of transferred cells may not behave as endogenous cells do [4, 55, 70].

**1.4.2 Tracking the endogenous CD8 T cell response to VV infection**

Tracking an endogenous CD8 response to a particular antigen is incredibly difficult unless the antigen can elicit a robust response capable of being detected with
current immunological methods, such as tetramers and peptide-stimulated intracellular cytokine staining (ICS). Recently, the immunodominant Kd-restricted epitopes of VV have been discovered [137]. Using these peptides to stimulate ex vivo CD8 T cells after vaccination allows the monitoring of an antigen-specific CD8 T cell response to VV when CD8 T cells are at their endogenous precursor frequencies. When combined with data from transgenic models, this model is instrumental in determining CD8 T cell response kinetics and cellular homing patterns.

1.5 Thesis Prospectus

The goal of vaccination is to provide effective protection against future pathogen challenge while stimulating the immune response against current infection. As vaccinia virus emerges as a powerful tool for vaccine development and immunotherapy, it is critical to understand the mechanisms by which vaccinia elicits a strong protective response. CD4 T cells have been shown to play a role in the generation of protective antibody responses to VV infection, but their role in promoting the CD8 T cell response is still largely undefined [138]. Full characterization of the function of CD4 T cells during VV infection is essential to its future clinical use since the loss of CD4 T cells is common in HIV-infected patients who progress to AIDS-related non-Hodgkin’s lymphoma [139]. Furthermore, intensive chemotherapy for sarcomas and non-Hodgkin’s lymphomas often results in an inability of CD4 T cells to repopulate the immune system [140].
overall goal of this thesis was to define the role of CD4 T cells in the CD8 T cell response to VV infection. To do this, we first sought to determine at which point CD4 T cells were required for optimal CD8 T cell primary and secondary responses. Next, we sought to determine the mechanism of CD4 T cell help both in vitro and in vivo as well as the signaling pathways activated by this help. We addressed this project with the following two chapters:

Chapter 3: At what point are CD4 T cells required for optimal CD8 T cell responses, and what role do they play?

Chapter 4: How is CD4 T cell help conferred, and what are the downstream signaling pathways of CD4 T cell help?
2. Materials and Methods

2.1 Mice

B10.D2 mice were purchased from the Jackson Laboratory. CD4-deficient mice (CD4\(^{-}\)) on the C57BL/6 background were purchased from the Jackson Laboratory and backcrossed onto the B10.D2 genetic background for nine generations. 129/Sv mice were obtained from Charles River Breeding Laboratories. STAT1\(^{-}\)/ mice on the 129/Sv background were purchased from Taconic. IL-21 receptor deficient mice (IL-21R\(^{-}\)) were provided by Dr. Warren Leonard (The National Institute of Health, Bethesda, MD) and backcrossed onto the B10.D2 background for nine generations. The Clone 4 HA-TCR transgenic mice that express a TCR recognizing a K\(^{d}\)-restricted HA epitope (518IYSTVASSL526) were kindly provided by Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA) [141]. The 6.5 TCR-HA transgenic mice that express a TCR recognizing an I-E\(^{d}\)-restricted HA epitope (110SFERFEIFPKE) were provided by Dr. H. von Boehmer (Harvard University, Boston, MA) [142]. These transgenic strains were backcrossed for more than nine generations onto the Thy1.1, B10.D2 genetic background. We intercrossed Clone 4 HA-TCR mice with IL-21R\(^{-}\) mice to generate the IL-21R\(^{-}\)/HA-TCR mice used in experiments. All mice used for experiments were between 6-8 weeks of age. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of the Duke University Medical Center.
2.2 Adoptive transfer of T cells

Naïve clonotypic HA-specific CD8+ T cells (Thy1.1) were prepared from Clone 4 TCR transgenic mice. Briefly, single cell suspensions were prepared from spleen and lymph nodes of Clone 4 TCR mice and clonotypic percentage was then determined by flow cytometry analysis of CD8+Vβ8.2+ cells as described [143, 144]. The activation marker CD44 was also checked to ensure these clonotypic cells were naïve. CD8 T cells were positively selected using anti-CD8 microbeads according to the manufacturer’s instructions (Miltenyi-Biotech) with a purity of >98%. Naïve polyclonal CD8+ T cells (Thy1.2) were prepared from either WT or IL-21R−/− mice. Briefly, single-cell suspensions were prepared from spleen and lymph nodes and CD8+ cells were positively selected using anti-CD8 microbeads according to the manufacturer’s instructions (Miltenyi Biotech). 1 x 10⁴ or 1 x 10⁶ purified CD8+ T cells were adoptively transferred to naïve recipients via tail vain injection in 200 μl HBSS. In some experiments cells were labeled with CFSE before transfer as previously described [143].

2.3 Immunizations and antibody treatment

Recombinant vaccinia virus encoding HA (rVV-HA) and recombinant E1-deleted adenovirus encoding HA (Ad-HA) were previously described [143]. rVV-HA was grown in TK-143B cells, purified by sucrose banding, and titer was determined by plaque forming assay on TK-143B cells. Mice were infected with 1x10⁷ PFU intravenously (i.v.) or 5 x 10⁵ or 5 x 10⁶ plaque-forming units (PFU) rVV-HA
intraperitoneally (i.p.). Ad-HA was grown in 293 cells (ATCC), purified by two rounds of CsCl density centrifugation and desalted by gel filtration through Sephadex G-25 column (PD-10 column, Amersham Bioscience). The titer was determined by plaque-forming assay on 293 cells. Mice were infected with \(2 \times 10^9\) PFU i.p.

*In vivo* CD4+ T cell depletion in B10.D2 mice was performed by i.p. injection of the anti-CD4 mAb GK1.5 (150 \(\mu\)g) for three days beginning 10 days before rVV-HA infection and every third day thereafter until completion of the experiment as described [103].

### 2.4 Antibodies and flow cytometry

Monoclonal antibodies (all from BD Biosciences unless indicated) used for staining were PE-Cy5-conjugated anti-CD8; FITC-conjugated anti-B220, -CD8, -CD44, -CD69, -CD62L, -CD122, -Granzyme B (eBiosciences), -IFN-\(\gamma\), -TNF-\(\alpha\), -Thy1.1, -Thy1.2, -pSTAT1, -pSTAT3, and -pSTAT5\(\alpha\); PE-conjugated anti-CD11c, -Thy1.1, -Thy1.2, Annexin V, and anti-Bcl-XL (Santa Cruz Biotechnology); Biotin-conjugated anti-CD127 and -Thy1.2, and APC-conjugated streptavidin. Collection of flow cytometry data was carried out using a FACScan or FACSCanto (BD Biosciences) and events were analyzed using CellQuest or FACSDiva software (BD Biosciences).
2.5 Intracellular staining

2.5.1 Intracellular cytokine staining

To assess production of effector molecules, splenocytes were cultured in 200 μl CTL medium (RPME-1640 supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 50 μM β-mercaptoethanol) at a concentration of 10⁷ cells/ml in the presence of 2 μg/ml of the Kd HA518-526 peptide and 5 μg/ml Brefeldin A containing Golgi-Plug (BD Biosciences) for 6 hr at 37 °C. After incubation, cells were washed and stained with anti-CD8 and anti-Thy1.1. Cells were then permeabilized using the Cytofix/cytoperm kit (BD Biosciences) and subsequently stained intracellularly with anti-IFN-γ, anti-TNF-α, or anti-Granzyme B.

2.5.2 Intracellular transcription factor staining

To measure intracellular levels of Bcl-xL, pSTAT1, and pSTAT3, cells were fixed with 3.7% formaldehyde, permeabilized with 90% methanol, blocked with 3% FBS, and subsequently stained with anti-CD8, -Thy1.2, and -Bcl-xL. For ex vivo detection of pSTAT1 and pSTAT3, splenocytes were stimulated for 4 hours in 200 μl of CTL medium (RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 50 μM 2-ME) at a concentration of 10⁷ cells/ml in the presence of 50 μg/ml PMA and 100 μg/ml Ionomycin before fixing.
2.6 Cell Isolation

2.6.1 Memory CD8 T cell isolation

Purified Clone 4 CD8 T cells were adoptively transferred into naive mice as described above. 45 days post rVV-HA infection, mice were sacrificed and spleen, superficial lymph nodes, and mesenteric lymph nodes were pooled. Cells were stained with PE-conjugated anti-Thy1.1 and FITC-conjugated anti-CD8. Thy1.1⁺ T cells were positively selected using anti-PE beads according to the manufacturer’s instructions (Miltenyi-Biotech). Enriched Thy1.1⁺ cells were then subjected to cell sorting gated on Thy1.1⁺CD8⁺ with a high speed FACS Vantage cell sorter (BD Biosciences). The purity of FACS sorted populations of cells was >95%.

2.6.2 Isolation of lymphocytes from non-lymphoid tissues

Lymphocytes were isolated from non-lymphoid tissues as described [54]. Briefly, liver or lung issue was homogenized and passed through a 70μm cell strainer. The single cell suspension was resuspended in 35 ml HBSS and centrifuged on a 15 ml Ficoll gradient (Amersham). Cells were harvested from the Ficoll gradient and washed twice with HBSS prior to analysis.

2.6.3 Activated DC isolation

For DC stimulation of naïve T cells, spleens were harvested from either uninfected or mice that had been infected for 24 hours with 1x10⁷ rVV-HA intravenously. Spleens were perfused with 417 μg/ml Liberase (Roche) and 5 μg/ml...
DNase (Sigma) for 30 minutes at 37°C. Spleens were then homogenized and passed through a 70-µm cell strainer. The single cell suspension was stained with PE-Cy5-conjugated anti-CD8, PE-conjugated anti-CD11c, and FITC-conjugated anti-B220 and subjected to cell sorting gated on CD8<sup>+</sup>CD11c<sup>+</sup>B220<sup>-</sup> with on a FACSDiVa (BD Biosciences) to over 95% purity. Naïve WT or IL-21R<sup>−/−</sup> HA-TCR CD8 cells were purified by positive selection using anti-CD8 beads, and naïve WT HA-TCR CD4 cells were purified by positive selection using anti-CD4 beads (Miltenyi Biotech).

**2.7 Stimulation of T cells in vitro**

**2.7.1 DC-mediated stimulation**

Sorted activated or naïve DCs were cultured with either CFSE-labeled or unlabeled naïve HA-TCR CD8 cells at a 1:10 ratio in 200 µl complete RPMI media. HA-TCR CD4 cells were added at equal numbers to CD8 cells.

**2.7.1 Antibody-mediated stimulation**

Polyclonal WT, IL-21R<sup>−/−</sup>, and STAT1<sup>−/−</sup> CD8 T cells were purified using anti-CD8 beads, and polyclonal CD4 T cells were purified using anti-CD4 beads (Miltenyi Biotech). For antibody stimulation of naïve T cells, 2x10⁵ cells were culturally in the presence of 1 µg/ml soluble anti-CD3 and 5 µg/ml soluble anti-CD28 (BD Biosciences) in 200 µl complete RPMI. CD4 cells were added at a 1:1 ratio with CD8 T cells. After 36 hours of stimulation, cells were washed and resuspended in 200 µl media for a total of 4
days. Recombinant murine IL-21 (R&D Systems) was added to cultures at a final concentration of 10 ng/ml.

2.8 Real-time quantitative PCR

Total RNA was isolated from purified cells using TRIzol reagent (Invitrogen), and cDNA was generated using a reverse transcription kit (Promega). Real-time PCR was performed using an iCycler (Bio-Rad) to measure SyBR green incorporation. The following primer sets were used: Bcl-XL, 5’-TGGTGATCCTCTCCCTCC-3’, 5’-CTCCATCCCGAAAGAGTTCA-3’; TRAIL, 5’-TCACCAACGAGATGAAGCAG-3’, 5’-GGCTAAGGTTTCCATCCATCC-3’; IL-2, 5’-CCACTCAAGCTCCACTCC-3’, 5’-ATCCTGGGGAGTTTTCAGGT-3’; IL-7, 5’-ATCCTTGTTCTGCTGCCTGT-3’, 5’-ACCAGCTTTGTGCTGCCTGT-3’; IL-15, 5’-GAGGCTGGGACCTGGG-3’, 5’-GCAATTCCAGGAGAAAGCAG; IL-21, 5’-CCCTTGCTCTGCTGCCTGT-3’, 5’-TGTTCCTCCCTCCCTCCCT-3’; Bcl-2, 5’-GACGTTAGCGAGAGAAGAG-3’, 5’-AAGCTGTCACAGAGGGCTA-3’; and Bim, 5’-GCCCTACCTCCTCCCTACAGAC-3’, 5’-CGCAGATCTCTCAGGTTCTCC-3’. Amounts of mRNA were normalized to hypoxanthine phosphoribosyltransferase (HPRT) or β-actin RNA levels within each sample.

2.9 Ovary vaccinia virus titer assay

Viral load in the ovaries was measured by plaque-forming assay as previously described [145]. 10⁴ purified Clone 4 CD8 T cells were transferred into female mice that
were subsequently infected with $5 \times 10^5$ PFU rVV-HA i.p. Mice were sacrificed 3 or 28 days post-infection and ovaries were harvested and stored at -80°C. Ovaries from individual mice were homogenized and freeze-thawed three times. Serial dilutions were performed and the viral titers were determined by plaque assay on confluent TK-143B cells.

### 2.10 Retrovirus preparation and infection

pMSCV constructs encoding GFP and GFP with dominant-negative STAT3 (STAT3-D), generously provided by Dr. Daniel Link (Washington University, St. Louis, MO), were used to produce recombinant retroviruses using BOSC 23 cells as described previously [146]. Briefly, ten micrograms of plasmid were transfected into BOSC 23 cells using calcium chloride-mediated transfection [147]. After three days, freshly prepared viral supernatants were added to cultures in the presence of $8 \mu g/ml$ polybrene 24 hours after polyclonal CD8 T cells were stimulated with $1 \mu g/ml$ anti-CD3 and $5 \mu g/ml$ anti-CD28. After 24 hours at 37°C, the retroviral supernatants were removed and replaced with CTL medium with or without murine IL-21 (10 ng/ml) and cells were cultured for an additional 72 hours. For RNA isolation, GFP+ cells were sorted by FACS using a MoFlo sorter (Beckman Coulter).

### 2.11 Statistical Analysis

Results were expressed as mean ± s.d. Differences between groups were examined for statistical significance using the student $t$-test.
3. CD4 T cells are required for CD8 T cell survival during both primary and memory recall responses

The following text was slightly modified from its original manuscript “CD4 T Cells Are Required for CD8 T Cell Survival during Both Primary and Memory Recall Responses,” published in Volume 179 of The Journal of Immunology [148].

3.1 Introduction

Adaptive CD8 T cell immunity represents an essential arm of the immune system to protect against many viral and bacterial infections [13, 149]. The course of the CD8 T cell response after an acute infection consists of three well-defined phases: clonal expansion of antigen-specific T cells to produce large numbers of effector cells, subsequent contraction of the majority (90-95%) of these effectors via apoptosis, and eventual development of a stable memory population from the surviving cells [149]. It has been shown that the clonal expansion and the contraction phases are programmed and that a brief encounter with antigen is sufficient to drive differentiation of naive CD8 T cell precursors into long-lived memory CD8 T cells [27, 61, 62, 150]. Although initial recognition of peptide-MHC by the TCR on naive CD8 T cells along with co-stimulation provided by mature dendritic cells is critical for induction of CD8 T cell responses following infection, other factors including CD4 T cells, have been shown to play an important role in influencing this process.
The importance of CD4 T cell help in primary CD8 T cell responses *in vivo* was first demonstrated in immunizations with noninflammatory antigens such as male minor HY antigen and alloantigen Qa-1 [87, 151]. Subsequent studies have shown that CD4 T cell help is required for the induction of optimal primary CD8 responses with soluble proteins, tumor antigens, and peptide-pulsed antigen-presenting cells (APCs) [89, 152, 153]. It is believed that in order to prime a CD8 T cell response in the absence of inflammation, APCs such as dendritic cells (DCs) have to be activated by CD4 T cells through CD40-CD40L interactions between DCs and CD4 T cells [94, 154, 155]. CD4 T cell help may also be provided by direct CD40-CD40L interactions between CD8 and CD4 T cells [122]. On the other hand, the primary CD8 T cell response against infectious pathogens was initially thought to be largely independent of CD4 T cell help [72, 96, 97]. This is because pathogens can provide the inflammatory stimuli such as Toll-like receptor (TLR) ligands and induce the production of inflammatory cytokines required for full activation of APCs, and thus bypass the need for CD4 T cell help [72]. However, recent studies have shown that primary CD8 T cell response to some pathogens, such as adenovirus [103], influenza virus [101], herpes simplex virus 1 (HSV-1) [102], and *Listeria monocytogenes* [100], is dependent on CD4 T cells. Thus, the nature of CD4 T cell help for primary CD8 T cell responses in the setting of infections remains to be defined.

Although the primary CD8 T cell response to infections can be independent of CD4 T cell help, recent studies have indicated that CD4 T cell help is required for the
generation of long-lived, functional memory CD8 T cells that respond rapidly upon secondary exposure to pathogens [98, 120, 121]. However, it remains controversial with regard to when the CD4 T cell help is needed for the generation of functional memory CD8 T cells. It has been suggested in some studies that CD4 T cell help during initial priming phase delivers the necessary 'instructive' signals for the generation of a fully functional memory CD8+ T cell pool [98, 120, 122]. In contrast, other studies have suggested that signals derived from CD4 T cells are required for regulating homeostasis of the memory CD8 T cells [99]. Furthermore, other observations have shown that the requirement for CD4 T cell help in memory CD8 T cell maintenance and function might be pathogen-specific [100, 101].

In this study, we sought to better understand when CD4 T cell help is required for the primary and memory CD8 T cell responses using a murine model of vaccinia virus (VV) infection. Here we found that the clonal expansion of antigen-specific CD8 T cells was severely compromised in CD4-deficient (CD4−/−) mice or wild type (WT) mice depleted of CD4 T cells. The reduced clonal expansion of CD8 T cells was not caused by a defect in T cell activation or proliferation, but rather by poor survival of activated T cells, suggesting CD4 T cell help is crucial for the survival of CD8 T cells during the primary response. As a result, a much smaller, but relatively stable CD8 memory pool was generated in the absence of CD4 T cells. Furthermore, we observed that in addition to CD4 T cell help provided during the primary response, the
“help” provided following a secondary challenge was also required for the survival of memory CD8 T cells during the recall expansion. These results suggest that CD4 T cell help is crucial for multiple stages of CD8 T cell response to VV infection. As VV has been used widely as vaccine vehicles for infectious diseases and cancer, our findings may have important implications for the design of effective vaccine strategies.

### 3.2 Results

#### 3.2.1 CD4 T cell help is needed for clonal expansion of CD8 T cells during the primary response to VV infection

To better understand the role of CD4 T cells in primary and secondary CD8 T cell responses to infection, we utilized a model where we could monitor the HA-specific CD8 T cell response to recombinant VV encoding HA (rVV-HA) *in vivo*. 10⁴ naïve Clone 4 HA-specific CD8 T cells (Thy1.1⁺) purified from Clone 4 HA-TCR transgenic mice that express a TCR recognizing a Kd-restricted HA epitope, were transferred into either wild type (WT) or CD4-deficient (CD4⁻/⁻) B10.D2 mice (Thy1.2⁺) that were subsequently infected with 5 x 10⁵ PFU rVV-HA intraperitoneally. 7 days after infection, splenocytes were analyzed for clonal expansion and effector differentiation of the Clone 4 CD8 T cells. Massive clonal expansion and effector differentiation as measured by the production of IFN-γ were detected in WT mice (Fig. 1A & B). By contrast, the extent of clonal expansion was significantly (p < 0.001) diminished when Clone 4 CD8 T cells were transferred into CD4⁺ mice (Fig. 1). A similar degree of reduction in clonal expansion
was found in other lymphoid and non-lymphoid organs including peripheral lymph nodes (LN), Peyer’s patch, liver and lung (Fig. 1B).

A recent report has shown that in CD4^{-/-} mice, the endogenous CD8 T cell population contains a large fraction of MHC class II-restricted cells [156]. To ensure the results seen in Fig. 1 were not due to a difference in the endogenous T cell repertoire, we performed the experiments in WT mice depleted of CD4 T cells using the depleting anti-CD4 mAb, GK1.5, prior to adoptive transfer of Clone 4 CD8^{+} T cells. Again, 10^{4} purified Clone 4 CD8 T cells were adoptively transferred into either untreated (WT) or GK1.5-treated (+GK1.5) B10.D2 mice that were subsequently infected with 5 \times 10^{5} PFU rVV-HA. 7 days later, splenocytes were harvested for analysis. As in CD4^{-/-} mice, Clone 4 CD8 T cells in the CD4-depleted mice had a significant (p <0.001) reduction in clonal expansion compared to the untreated WT mice (Fig. 1). Collectively, these results indicate that CD4 T cell help is critical for clonal expansion during the primary response to VV infection in vivo.
Figure 1: Defective clonal expansion of CD8 T cells in the absence of CD4 T cell help during a primary response to VV infection. 10^4 purified naïve Clone 4 CD8 T cells (Thy1.1+) were adoptively transferred into congenic wild type (WT), CD4-deficient (CD4−) B10.D2 mice (Thy1.2+), or WT mice treated with the depleting CD4 mAb GK1.5 (+GK1.5), which were subsequently infected with rVV-HA. 7 days later, spleen and other lymphoid and non-lymphoid organs were harvested for analysis of transferred cells. (A) Expansion and function of clonotypic cells. Splenocytes were stained with anti-CD8, anti-Thy1.1 and anti-IFN-γ intracellularly. Percentage of total (left panels) and IFN-γ-producing (right panels) clonotypic cells among total lymphocytes is indicated with the numbers in parentheses showing the mean fluorescence intensity (MFI, x10^2) of IFN-γ-producing clonotypic cells. (B) The mean absolute numbers of clonotypic T cells per spleen, combined 6 peripheral LNs, combined 6 Peyer’s patches, whole liver or whole lung are indicated with standard deviations. Data shown are representative of three independent experiments.
3.2.2 CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help

We next investigated what contributed to the defect in CD8 T cell expansion during the primary response to VV infection in the absence of CD4 T cells. One possibility is that CD8 T cells are not fully activated without CD4 T cell help. To address this, we transferred $10^6$ naïve Clone 4 CD8 T cells into WT, GK1.5-treated or CD4−/− mice and subsequently infected the hosts with $5 \times 10^6$ PFU rVV-HA. Higher Clone 4 T cell numbers ($10^6$) were used due to the fact that $10^4$ transferred cells were below the limit of detection at early time points. 24 hr after infection, Clone 4 CD8 T cells in WT, GK1.5-treated, and CD4−/− mice displayed a similarly activated phenotype of CD44high and CD69high compared to the naïve CD8 T cell phenotype of CD44low and CD69low (Fig. 2A). 3 days after infection, CFSE-labeled Clone 4 CD8 T cells in WT, GK1.5-treated, and CD4−/− mice underwent several rounds of division similarly by CFSE dilution (Fig. 2B), suggesting CD8 T cell proliferation was also not affected by a lack of CD4 T cell help. Furthermore, despite a reduced clonal size, the effector differentiation of Clone 4 CD8 T cells in both GK1.5-treated and CD4−/− mice appeared to be intact at day 7 after infection as the production of IFN-γ on a per cell basis (as measured by MFI) was similar to that in WT mice (Fig. 1A). Similarly, the production of other effector molecules such as TNF-α and Granzyme B appeared to be normal in GK1.5-treated and CD4−/− mice compared to that in WT mice (Fig. 2C). Additionally, the phenotype of effector CD8 T cells as measured by CD62L downregulation, CD122 upregulation, and CD127 re-upregulation
was not affected by the lack of CD4 T cells in both the GK1.5-treated and the CD4+/ hosts as compared to WT mice (Fig 2D). These data suggest that CD8 T cell activation and effector differentiation in response to VV infection in vivo is not affected by a lack of CD4 T cell help.
Figure 2: CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help.
Figure 2: CD8 T cell activation and effector differentiation in response to VV infection is not affected by a lack of CD4 T cell help. (A-B) 10⁶ purified naïve Clone 4 CD8 T cells were transferred to WT, CD4⁻/⁻ mice, or WT mice treated with GK1.5 mAb (+GK1.5) that were subsequently infected with rVV-HA. Some WT mice were left uninfected (Naïve). (A) 24 hr post-infection, splenocytes were harvested and stained with antibodies against CD8, Thy1.1, and the activation markers CD44 or CD69. The percentages of CD44³⁺ and CD69³⁺ are indicated. (B) 3 days post-infection, in vivo division of CFSE-labeled clonotypic cells in the spleen was analyzed. (C-D) 10⁴ purified naïve Clone 4 CD8 T cells were transferred into different recipients that were subsequently infected with rVV-HA. 7 days later, splenocytes were harvested, stained with anti-CD8 and anti-Thy1.1 and analyzed for the production of effector molecules and the expression of surface markers. (C) The percentages of TNF-α and Granzyme B (GRB) producing clonotypic cells are indicated. (D) The percentages of CD62I⁺, CD122⁺⁺, and CD127⁺⁺ are indicated. All plots are gated on CD8⁺Thy1.1⁺ cells. Data shown are representative of three independent experiments.
3.2.3 The survival of activated CD8 T cells during priming is dependent on CD4 T cell help

Since CD8 T cell activation, proliferation and effector differentiation do not appear to be altered due to the lack of CD4 help, we then asked if the difference in clonal expansion could be due to decreased survival of the activated CD8 T cells in the absence of CD4 T cells. We used Annexin V staining to assess CD8 T cells undergoing apoptosis. 10⁴ naïve Clone 4 CD8 T cells were transferred into WT, GK1.5-treated, or CD4⁻/⁻ mice, followed by infection with 5 x 10⁵ PFU rVV-HA. 7 days after infection, mice were harvested for analysis. Indeed, activated Clone 4 CD8 T cells in both GK1.5-treated and CD4⁻/⁻ mice displayed a significant ($p<0.001$) increase in Annexin V positivity (51.1% and 55.7%, respectively) compared to WT mice (20.3%, Fig. 3A). This increased apoptosis of activated CD8 T cells in the absence of CD4 T cell help correlated with a significant ($p<0.001$) reduction in the expression of the pro-survival molecule, Bcl-xL at both the message RNA and protein levels (Fig. 3B,D). TRAIL expression has been implicated in regulating secondary expansion of the “helpless” memory CD8 T cells [125]. Here we showed that TRAIL expression was also significantly ($p<0.001$) upregulated in the activated CD8 T cells during primary response to VV infection in the absence of CD4 T cells (Fig. 3C). Taken together, these results suggest that the diminished clonal expansion of CD8 T cells in response to VV infection in the absence of CD4 T cells is not caused by a reduction in T cell activation, but by poor survival of activated CD8 T cells.
Figure 3: CD4 T cells promote the survival of activated CD8 T cells during priming in vivo. 10^4 purified naïve Clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or CD4^-/- mice that were subsequently infected with rVV-HA. Some WT mice were left uninfected (Naïve). 7 days later, splenocytes were harvested for subsequent analysis. (A) Annexin V staining. The percentage of Annexin V+ cells among clonotypic cells is indicated. (B-C) Clonotypic cells were purified by cell sorting and subjected to real-time quantitative PCR to measure the expression of Bcl-xL (B) and TRAIL (C). Data are presented as normalized mRNA abundance to hypoxanthine phosphoribosyltransferase. (D) Cells from different recipients were stained with Bcl-xL intracellularly or an isotype control Ab (Isotype). The mean fluorescence intensity (MFI) of clonotypic cells is indicated. Plots are gated on CD8^+Thy1.1^+ cells. Data shown are representative of three independent experiments.
3.2.4 Diminished, but relatively stable CD8 memory pool can develop in absence of CD4 T cells

We next determined the ability of effector CD8 T cells to develop into stable memory cells in the absence of CD4 T cell help. After the peak of clonal expansion at day 7, splenic Clone 4 effector CD8 T cells in the WT recipients underwent marked contraction between days 7 and 14, and those that survived developed into stable memory CD8 T cells (Fig. 4A, B). This is consistent with previous observations in other models of bacterial or viral infections [13, 27, 149]. Similarly, after contraction, Clone 4 effector CD8 T cells generated in both the GK1.5-treated and CD4−/− hosts were also capable of differentiating into memory cells, but with a significant (p <0.001) reduction in memory size that was proportional to the size of effectors (Fig. 4A, B). This reduction was not a result of differential homing of memory cells in the absence of CD4 T help as a similar degree of decrease was observed in other lymphoid and non-lymphoid organs such as peripheral lymph nodes (Fig. 4C), Peyer’s patch (Fig. 4D), and liver (Fig. 4E), in both the GK1.5-treated and CD4−/− mice. Neither was this decrease in the memory size due to a persistent viral infection, as viral titers performed on day 28 after infection showed that the virus was cleared in the GK1.5-treated and CD4−/− hosts as efficiently as WT mice (Table 1). Despite a reduction in their size, the memory CD8 T cells generated in the GK1.5-treated and CD4−/− hosts appeared relatively stable at least up to day 55 after infection (Fig. 4A). Furthermore, the production of the effector molecules IFN-γ, TNF-α, and Granzyme B, as well as the expression of the surface markers CD62L,
CD122, and CD127 appeared to be similar in the WT, GK1.5-treated, and CD4−/− mice (Fig. 5). Thus, a diminished, but relatively stable memory CD8 pool can develop following VV infection in the absence of CD4 T cell help.

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.15 x 10³ ± 212</td>
<td>0</td>
</tr>
<tr>
<td>+GK1.5</td>
<td>1.37 x 10³ ± 495</td>
<td>0</td>
</tr>
<tr>
<td>CD4−/−</td>
<td>1.2 x 10³ ± 282</td>
<td>0</td>
</tr>
</tbody>
</table>

A total of 1 x 10⁴ purified naïve Clone 4 CD8 T cells were transferred into WT, WT + GK1.5, or CD4−/− mice that were subsequently infected with 5 x 10⁵ PFU rVV-HA. Three or 28 days later, ovaries were harvested and lysate was used to determine viral titer by plaque assay.
Figure 4: Diminished, but relatively stable CD8 memory pool can develop in the absence of CD4 T cells. 10^4 purified naïve Clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or CD4^-/- mice that were subsequently infected with rVV-HA. (A) 7, 15, 28, and 55 days later, splenocytes were stained with anti-CD8 and anti-Thy1.1. The mean absolute numbers with standard deviations of clonotypic cells is indicated. (B-E) 55 days after infection, the mean absolute numbers with standard deviations of clonotypic cells per spleen (B), combined 6 peripheral LNs (C), combined 6 Peyer’s patches (D), and whole liver (E), are indicated. Data are representative of two independent experiments.
Figure 5: Phenotypic and function analyses of the “helpless” memory CD8 T cells. 10^4 purified naïve Clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or CD4^-/^- mice that were subsequently infected with rVV-HA. 55 days later, splenocytes were harvested for analyses. Percentages of IFN-γ^+, TNF-α^+, Granzyme B^+, (GRB), CD62L^high, CD122^high, and CD127^high are indicated. Plots are gated on CD8^+Thy1.1^+ cells. Data are representative of two independent experiments.
3.2.5 CD4 T cell help is also required for CD8 memory recall expansion following secondary challenge

One hallmark of memory cells is a rapid and more efficacious response upon secondary encounter with a pathogen. It is not entirely clear what controls this rapid recall potential of memory cells. Previous studies have suggested that CD4 T cell help during the primary response is needed for the generation of fully functional memory cells that can respond to secondary challenge rapidly [98, 120, 122]. However, it is less clear if CD4 T cells are needed following rechallenge for recall expansion. If so, what is the relative contribution of CD4 T cell help provided during the primary response vs. following rechallenge to the recall expansion? To address these questions, Clone 4 memory CD8 T cells were purified by FACS sorting from WT, GK1.5-treated, or CD4$^{-/-}$ mice 45 days after infection with rVV-HA. Equal numbers ($3.5 \times 10^4$) of purified memory cells were then transferred into naïve WT, GK1.5-treated, or CD4$^{-/-}$ mice that were subsequently challenged with $5 \times 10^6$ PFU rVV-HA intraperitoneally. 7 days after rechallenge, splenocytes were analyzed for the recall expansion and effector function of transferred memory cells. Vigorous recall expansion of Clone 4 CD8 T cells was detected in WT recipients that received memory cells from WT donors, whereas the extent of recall expansion was significantly ($p < 0.05$) reduced in WT recipients transferred with memory cells from either GK1.5-treated or CD4$^{-/-}$ donors (Fig. 6). This is consistent with the notion that CD4 T cell help during the primary response provides the necessary 'instructive' signals for the generation of fully functional memory cells [98, 120, 122]. To
our surprise, a much greater reduction in recall expansion ($p < 0.001$) was observed when memory cells from WT, GK1.5-treated, or CD4$^+$ donors were transferred into either CD4-depleted or CD4$^+$ recipients (Fig. 6). Similar results were obtained when mice were challenged with recombinant adenovirus expressing HA (data not shown). These results indicate that CD4 T cell help provided following secondary challenge is also critical for recall expansion of memory CD8 T cells, in addition to that provided during the primary response.
Figure 6: CD4 T cell help provided both during initial priming and following rechallenge is required for recall expansion of memory CD8 T cells.
Figure 6: CD4 T cell help provided both during initial priming and following rechallenge is required for recall expansion of memory CD8 T cells. Purified naïve Clone 4 CD8 T cells were transferred to WT, GK1.5-treated WT (+GK1.5), or CD4⁻/⁻ mice that were subsequently infected with rVV-HA. 45 days later, clonotypic memory CD8 T cells were purified by FACS sorting and 3.5 x 10⁴ memory cells from WT hosts were adoptively transferred into naïve WT (WT → WT), GK1.5-treated (WT → +GK1.5) or CD4⁻/⁻ (WT → CD4⁻/⁻) recipients. Similarly, equal numbers of memory cells from either GK1.5-treated or CD4⁻/⁻ hosts were transferred into WT (+GK1.5 → WT or CD4⁻/⁻ → WT), GK1.5-treated (+GK1.5 → +GK1.5), or CD4⁻/⁻ (CD4⁻/⁻ → CD4⁻/⁻) recipients. The recipients were subsequently challenged with rVV-HA. 7 days post-challenge, splenocytes were harvested and analyzed. (A) The mean absolute numbers of clonotypic CD8 T cells with standard deviations are indicated. (B) The percentages of IFN-γ-producing clonotypic CD8 T cells are indicated with the numbers in parentheses showing the mean fluorescence intensity (MFI) of IFN-γ-producing clonotypic CD8 T cells. Data shown are representative of three independent experiments.
3.2.6 CD4 T cell help promotes the survival of CD8 T cells during recall expansion

Despite the compromised recall expansion in the absence of CD4 T cell help either during the primary response or following rechallenge, the effector function of Clone 4 CD8 T cells after recall expansion appeared to be intact as their ability to produce IFN-γ on a per cell basis (as measured by MFI) was similar to that of “helped” WT control (Fig. 6B). This result suggested that the dependency of CD8 memory recall expansion on CD4 T cell help was likely mediated by promoting their survival, similar to our observations during the primary response. To address this question, Clone 4 memory CD8 T cells (3.5 x 10⁶) were purified from the WT, GK1.5-treated, or CD4⁻/⁻ donors 45 days after infection with rVV-HA and transferred into WT, GK1.5-treated, or CD4⁺ recipients that were subsequently infected with 5 x 10⁶ PFU rVV-HA intraperitoneally. 7 days later, splenocytes were analyzed for Clone 4 CD8 T cells that were undergoing apoptosis by Annexin V staining. A significant (p <0.001) increase in Annexin V⁺ cells was detected in WT recipients that received memory cells from either GK1.5-treated or CD4⁻/⁻ donors (44.1% and 54.6%, respectively) compared to WT donors (22.3%, Fig. 7). A further increase in Annexin V⁺ cells was observed when memory cells from WT, GK1.5-treated, or CD4⁻/⁻ donors were transferred into GK1.5-treated or CD4⁻/⁻ recipients (Fig. 7). These results indicate that indeed CD4 T cell help during the primary and secondary response promotes the survival of CD8 T cells during recall expansion.
Figure 7: The survival of memory CD8 T cells during a recall expansion is dependent on CD4 T cells. 3.5 x 10⁴ memory cells were purified from day 45 rVV-HA infected WT, GK1.5-treated (+GK1.5) WT, or CD4⁻/⁻ mice and adoptively transferred into naïve WT, GK1.5-treated (+GK1.5) WT, or CD4⁻/⁻ recipients that were subsequently infected with rVV-HA. 7 days post-challenge, spleens were harvested and stained with anti-CD8, anti-Thy1.1, and Annexin V. The percentage of Annexin V⁺ cells among clonotypic cells is indicated. Plots are gated on CD8⁺Thy1.1⁺ cells. Data shown is representative of three independent experiments.
3.3 Discussion

In this study, we have shown that CD4 T cell help plays a critical role in both primary and memory CD8 T cell responses to VV infection. We demonstrate that although CD4 T cell help is not needed for activation and effector differentiation of antigen-specific CD8 T cells during a primary CD8 T cell response to VV infection, the survival of activated CD8 T cells is dependent on CD4 T cell help, leading to a reduction in clonal expansion and a diminished, but stable CD8 memory pool. Furthermore, we also demonstrate that the “help” provided by CD4 T cells both during the primary response and following secondary challenge is required for recall expansion of memory CD8 T cells by promoting their survival.

It has been well documented that CD4 T cell help is important for the induction of primary CD8 T cell response to noninflammatory antigens such as minor histocompatibility antigens, tumor antigens or protein antigen in vivo [87, 89, 151-153]. This is achieved by activating or “licensing” the DCs through CD40-CD40L interactions between DCs and CD4 T cells [94, 154, 155], or via direct CD40-CD40L interactions between CD8 and CD4 T cells [122]. On the contrary, it had been initially thought that primary CD8 T cell response to infectious pathogens is largely independent of CD4 T cell help as pathogens can provide the inflammatory signals to promote full activation of DCs [72, 96, 97]. However, some primary CD8 T cell responses to pathogens such as adenovirus [103], influenza virus [101], herpes simplex virus 1 (HSV-1) [102], and Listeria
monocytogenes [100], are CD4 T cell help-dependent. Since direct CD40–CD40L interaction between CD8 and CD4 T cells is not involved in these infections [104, 105], it has been unclear why CD4 T cell help is needed in these settings. Our results presented here demonstrate that CD4 T cell help is also required for primary CD8 T cell response to VV infection in vivo. Consistent with the notion that pathogens can activate DCs directly for efficient T cell priming and thus bypass the need for CD4 T cell help [72], the activation and effector differentiation of CD8 T cells during the primary response to VV infection is independent of CD4 T cells. However, the survival of activated CD8 T cells is critically dependent on CD4 T cell help and as a result, the clonal expansion of antigen-specific CD8 T cells is diminished without CD4 T cell help.

How does CD4 T cell help promote the survival of activated, antigen-specific CD8 T cells during the primary response in vivo? It is possible that CD4 T cells could either directly provide survival signals to activated CD8 T cells or indirectly act on an intermediate cell that provides CD8 T cells with such signals. A recent report in vitro has implicated CD4 T cells in protecting activated CD8 T cells from activation-induced cell death (AICD) through a direct cell-to-cell contact mechanism [157]. Although AICD of CD4 T cells has been considered to be mediated by Fas-FasL interaction [158, 159], it remains controversial which death receptors are involved in AICD of CD8 T cells. Regulation of TRAIL expression by CD4 T cell help has been implicated in protecting memory CD8 T cells from AICD during a recall expansion [125]. However, a recent
study has suggested that CD4 T cell help consists of both TRAIL-dependent and – independent mechanisms [160]. In line with these observations, we provided evidence that in the absence of CD4 T cell help, TRAIL expression is upregulated in the activated CD8 T cells during the primary response to VV infection. In addition, there is a significant reduction in the expression of the pro-survival molecule, Bcl-XL in the “helpless” CD8 T cells, suggesting that the intrinsic apoptotic pathway [161] may also be involved in CD4 T cell-mediated protection of activated CD8 T cells from AICD *in vivo*. Thus, future studies will be needed to elucidate the protective signals that CD4 T cells provide, and the signaling pathway(s) involved in promoting the survival of activated CD8 T cells during the primary response *in vivo*.

Despite the poor survival of activated CD8 T cells without CD4 T cell help during priming, which leads to a reduction in clonal expansion, these “helpless” effector CD8 T cells can develop into relatively stable memory cells albeit with a diminished memory size that is proportional to the size of effector T cells. This suggests that after contraction phase, the maintenance of memory CD8 T cells after VV infection is independent of CD4 T cells. This is in contrast to the previous observation that the maintenance of memory CD8 T cells after an acute infection with LCMV is compromised in MHC class II-deficient mice that lack CD4 T cells [99]. The reasons for the discrepancy are not clear, but could be related to the pathogens used for the experiments. Indeed, recent studies have shown that the requirement for CD4 T cell help in memory CD8 T
cell maintenance might be pathogen-specific [100, 101]. We have further observed that the “helpless” memory CD8 T cells are similar to the “helped” ones phenotypically as measured by the expression of CD62L, CD122, and CD127, as well as functionally in terms of the production of the effector molecules such as IFN-γ, TNF-α, and Granzyme B. This is in contrast to a previous report with LCMV that the “helpless” memory CD8 T cells showed a CD62LlowCD122low phenotype, suggesting a defect in the formation of CD62Lhigh central memory cells [162]. Again, it is not clear what contributes to the differences, but might be pathogen-related.

The requirement for CD4 T cells in promoting fully functional memory CD8 T cells that can respond rapidly upon secondary challenge has been well studied [98, 120-122]. However, the majority of studies have focused on the CD4 T cell help provided during initial priming phase, which delivers the necessary 'instructive' signals for the generation of fully functional memory CD8+ T cells. It is less clear whether CD4 T cells are also needed following secondary challenge for the rapid recall expansion. Consistent with the previous observations, we have shown in this study that indeed CD4 T cell help is required during the primary response to VV infection for the generation of rapid recall response. We have also demonstrated that the presence of CD4 T cells following a secondary challenge is also crucial to the recall expansion of memory CD8 T cells. Our results are in contrast to the observations by Shedlock and Shen [120]. In their study, only CD4 T cell help provided during initial priming with VV encoding GP33-41 epitope
from LCMV was important for recall expansion following a secondary challenge. However, LCMV was used for the secondary challenge instead of VV. Since VV has been used extensively as vaccine vehicles for infectious diseases and cancer, our results may be more relevant to the design of effective vaccine strategies.

We have also provided evidence that defective recall expansion in the absence of CD4 T cell help either during the primary response or following rechallenge is due to poor survival. Similar to the requirement of CD4 T cells for the survival of CD8 T cells during the primary response, the mechanism(s) underlying the dependency of memory CD8 T cell survival on CD4 T cell help during recall expansion remains to be defined. Although TRAIL expression has been implicated in regulating the memory CD8 T cells that lack CD4 T cell help during initial priming from AICD during a recall expansion [125], it is not clear if the same mechanism applies to the CD4 T cell help provided following secondary challenge. Future studies are needed to delineate the exact mechanism(s) by which CD4 T cells promote the survival of memory CD8 T cells during a recall expansion in vivo.

In summary, we have demonstrated that CD4 T cells are crucial to both primary and memory CD8 T cell responses to VV infection. This is achieved by promoting the survival of antigen-specific CD8 T cells during the initial priming and the recall expansion following rechallenge. As one major goal of vaccination is to maximize the magnitude of CD8 T cell response and to generate fully functional memory CD8 T cells,
our results may have important implications for the design of effective strategies for treating infectious diseases and cancer.
4. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection

The following text was slightly modified from its original manuscript “Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection,” currently under review at The Journal of Experimental Medicine.

4.1 Introduction

The course of the CD8 T cell response after an acute infection or vaccination consists of three distinct phases: clonal expansion and development of effector functions, subsequent contraction of the majority of effector T cells via apoptosis, and generation of long-lived memory cells from the surviving cells [13, 27, 149]. The signals that regulate the survival of effector CD8 T cells and promote the generation of memory CD8 T cells remain largely unknown. Studies have shown that CD4 T cell help plays an important role in influencing these processes [72]. Although the primary CD8 T cell response against infectious pathogens can be CD4 T cell help-dependent [100-103] or -independent [72, 97], CD4 T cell help is essential for the generation of long-lived, functional memory CD8 T cells that respond rapidly upon secondary exposure to pathogens [98, 120, 121]. Indeed, we have recently demonstrated that CD4 T cell help is also critical for CD8 T cell responses to an acute infection with vaccinia virus (VV) [148]. This is achieved by promoting the survival of activated CD8 T cells during both the
primary and memory recall responses [148]. Similar observations were made in a model of *Listeria monocytogenes* infection [100]. However, it remains to be defined how CD4 T cell help promotes CD8 T cell survival during the primary and recall responses to pathogens.

IL-21 is the most recently identified member of the common-γ chain family of cytokines that includes IL-2, IL-4, IL-7, IL-9, and IL-15 [112, 163]. This cytokine is mainly produced by activated CD4 T cells [163], including follicular helper T (Tfh) cells [164], as well as NKT cells [111], and plays a critical role in the control of both innate and adaptive immune responses [112]. IL-21 can regulate NK cell activation and expansion [163, 165] and promote terminal B cell differentiation into plasma cells, which is critical for antibody production [166, 167]. IL-21 can also enhance resting T cell proliferation *in vitro* in combination with IL-7 or IL-15 and promote antigen-specific CD8 T cell expansion *in vivo* [116]. In addition, it is critical for the development of Tfh cells [164] and the inflammatory Th17 lineage [168, 169] and also contributes to autoimmunity [112]. More recently, IL-21 has been shown to be an essential component of CD4 T cell help required to sustain the CD8 T cell response during chronic, but not acute, LCMV infections [117-119]. This is achieved by direct action of IL-21 on CD8 T cells to avoid deletion and maintain immunity. However, two major questions remain: 1) The role of IL-21 in the CD8 T cell response to CD4 T cell help-dependent acute infections such as
VV infection is still unknown; and 2) The mechanism(s) underlying the cell-intrinsic, IL-21-dependent enhancement of CD8 T cell immunity is yet to be defined.

This study provides the first direct evidence that CD4 T cell help for CD8 T cell survival was mediated by IL-21. We then demonstrated that direct IL-21 signaling on CD8 T cells was required for the priming of VV-specific CD8 T cell response in vivo. Using clonotypic influenza hemagglutinin (HA)-specific transgenic T cells, we found that the activation, proliferation or effector differentiation of CD8 T cells in response to VV infection in vivo was not affected by lack of IL-21 signaling. However, the survival of effector CD8 T cells was critically dependent on intrinsic IL-21 signaling. Furthermore, CD8 T cells deficient in IL-21 signaling failed to develop into long-lived memory cells. We further observed that IL-21 promoted CD8 T cell survival by activating the STAT1 and STAT3 signaling pathways and subsequent upregulation of the pro-survival molecules, Bcl-2 and Bcl-xL. In vivo, CD8 T cells defective for IL-21 signaling had reduced levels of STAT1 and STAT3 activation and Bcl-xL upregulation in response to VV infection. Collectively, our study indicates that intrinsic IL-21 signaling is required for the survival of activated CD8 T cells and the formation of long-lived memory cells in response to VV infection and may have important implications in the design of effective vaccine strategies.
4.2 Results

4.2.1 CD4 T cell help for CD8 T cell survival is mediated by IL-21

To study the mechanism(s) by which CD4 T cell help promotes CD8 T cell survival, we first looked at the role of CD4 T cell licensing of dendritic cells (DCs) in CD8 T cell immunity since this has been shown to be important in HSV-1 infection [102]. We utilized a previously described model where conventional CD8+ DCs from pathogen-infected mice can be used to prime naïve CD8 T cells in vitro [170]. Wild-type (WT) or CD4-deficient (CD4−/−) mice were infected with recombinant VV encoding influenza hemagglutinin (rVV-HA), and 24 h later, CD8+CD11c−B220− DCs were purified and used to stimulate naïve HA-specific CD8 T cells derived from the Clone 4 HA-TCR transgenic mice that express a TCR recognizing a Kd-restricted HA epitope in vitro. 4 days later, similar extents of CD8 T cell proliferation measured by CFSE dilution (Fig. 8A) and apoptosis detected by Annexin V staining (Fig. 8B) were observed with DCs derived from either rVV-HA infected WT or CD4−/− mice, suggesting that CD4 T cell licensing of DCs during VV infection is not important in promoting CD8 T cell proliferation or survival.

We then examined whether CD4 T cell help is contact-dependent, as recent data have suggested that direct cell-to-cell contact between CD4 and CD8 T cells may protect CD8 T cells from activation-induced cell death [157]. To address this, we utilized a culture system where naïve polyclonal CD8 T cells are either mixed with naïve CD4 T
cells or separated from them in a transwell, followed by stimulation with soluble anti-CD3 and anti-CD28 mAbs for 4 days. Our data showed that while CD4 T cells did not enhance CD8 T cell proliferation (Fig. 8C), they markedly enhanced CD8 T cell survival whether mixed together or separated by a transwell (Fig. 8D), indicating that CD4 T cell help for CD8 T cell survival is mediated by a soluble factor in vitro.

We next determined what soluble factor from CD4 T cells enhanced CD8 T cell survival. Since the common-γ chain cytokines IL-2, IL-7, IL-15, and IL-21 have been shown to be the key regulators of CD8 T cell responses [112, 171], we examined which of these cytokines were upregulated in the activated CD4 T cell population by real-time PCR. Both IL-2 and IL-21 mRNA levels were increased in activated CD4 T cells (Fig. 9A). Since IL-2 has been shown to be dispensable for CD8 T cell survival in vivo [123], we tested whether IL-21 could play a role in the survival of activated CD8 T cells. The addition of recombinant IL-21 to polyclonal CD8 T cells that were stimulated with anti-CD3 and anti-CD28 enhanced their survival in vitro: an effect that was dependent on the presence of the IL-21 receptor (IL-21R) on the CD8 T cells (Fig. 9B).

To determine whether CD4 T cell-derived IL-21 enhances CD8 T cell survival, we intercrossed the IL-21R-deficient (IL-21R−/−) mice with the Clone 4 HA-TCR mice to generate IL-21R−/− Clone 4 T cells. We then cultured either WT or IL-21R−/− Clone 4 CD8 T cells with DCs derived from rVV-HA infected WT mice in the presence or absence of HA-specific CD4 T cells from the 6.5 TCR-HA mice that express a TCR recognizing an I-
E^4-restricted HA epitope. We found that the enhancement of CD8 T cell survival by CD4 T cells was abolished in the absence of IL-21 signaling (Fig. 9C). Taken together, these results suggest that CD4 T cell help for the survival of activated CD8 T cells is mediated by direct IL-21 signaling on CD8 T cells in vitro.
Figure 8: CD4 T cell help for CD8 T cell survival is mediated by a soluble factor in vitro. (A-B) WT or CD4⁻/⁻ mice were infected with rVV-HA (1 x 10⁷ PFU) intravenously, or left uninfected (Naive). 24 h later, CD8⁺CD11c⁻B220⁻ DCs were purified by FACS, and cultured with purified naïve Clone 4 HA-specific CD8 T cells for 4 days. (A) Division of CFSE-labeled clonotypic CD8 T cells. (B) Annexin V staining. Percentage of Annexin V⁺ clonotypic CD8 T cells is indicated. (C-D) Polyclonal CD8 T cells were stimulated with soluble anti-CD3 and anti-CD28 or left unstimulated for 4 days. In some wells, polyclonal CD4 T cells were added by either mixing with the CD8 T cells or placing in a transwell support. (C) Division of CFSE-labeled CD8 T cells. (D) Annexin V staining. Percentage of Annexin V⁺ CD8 cells is indicated. All plots are gated on CD8⁺ T cells. Data are representative of three independent experiments.
Figure 9: CD4 T cell help for the survival of activated CD8 T cells in vitro is mediated by IL-21.
Figure 9: CD4 T cell help for the survival of activated CD8 T cells in vitro is mediated by IL-21. (A) Polyclonal CD4 T cells were stimulated with soluble anti-CD3 and anti-CD28 or left unstimulated. 36 h later, RNA was extracted and real-time quantitative PCR was used to measure the expression of IL-2, IL-7, IL-15, and IL-21. mRNA abundance was normalized to β-actin. Data are presented as the fold increase of the corresponding cytokine mRNA relative to that of unstimulated cells. (B) Polyclonal WT or IL-21R−/− CD8 T cells were stimulated with soluble anti-CD3 and anti-CD28 or left unstimulated as a control for 4 days. Recombinant murine IL-21 was added where indicated. Cells were stained with anti-CD8 and Annexin V. Percentages of Annexin V−CD8+ T cells are indicated. (C-D) CD8−CD11c−B220− DCs were purified from uninfected (Naive) and rVV-HA infected (rVV-HA) spleens and cultured for 4 days with purified naïve Clone 4 HA-specific CD8 T cells from WT or IL-21R−/− Clone 4 HA-TCR mice. Purified naïve 6.5 HA-specific CD4 T cells were added where indicated. (C) Annexin V staining. Percentage of Annexin V− clonotypic CD8 T cells is indicated. Data are representative of three independent experiments.
4.2.2 The priming of VV-specific CD8 T cells *in vivo* is dependent on intrinsic IL-21 signaling

We next asked whether this intrinsic IL-21 signaling is required for the CD8 T cell response to VV infection. To address this, we transferred polyclonal CD8 T cells from WT or IL-21R<sup>−/−</sup> mice (Thy1.2<sup>+</sup>) into congenic B10.D2 recipients (Thy1.1<sup>+</sup>) that were subsequently vaccinated with rVV-HA. 7 days later, splenocytes were analyzed for VV-specific CD8 T cell response by intracellular IFN-\(\gamma\) staining upon re-stimulation *in vitro* with the \(K^d\)-restricted VV F2L epitope immunodominant peptide [137]. We found that the percentage and the absolute cell number of the F2L-specific CD8 T cells were significantly \((p<0.001)\) reduced in the transferred IL-21R<sup>−/−</sup> CD8 T cells, compared to the transferred WT controls (Fig. 10A, B). However, the endogenous CD8 T cell response to VV F2L is comparable in both recipients (Fig. 10A). When the transferred CD62L<sub>low</sub> (activated) CD8 T cells are stained for Annexin V, IL-21R<sup>−/−</sup> cells displayed a significant \((p<0.001)\) increase in Annexin V positivity (43.8%), compared to WT cells (18.7%, Fig. 10C, D). These data suggest that the priming and survival of VV-specific CD8 T cells is dependent on IL-21 signaling.
Figure 10: Intrinsic IL-21 signaling is required for the priming of VV-specific CD8 T cells in vivo. 1 x 10⁷ purified polyclonal naïve CD8 T cells (Thy1.2⁺) from either WT or IL-21R⁻ mice were adoptively transferred into congenic B10.D2 mice (Thy1.1⁺) that were subsequently infected with rVV-HA (5 x 10⁶ PFU, i.p.). 7 days later, splenocytes were harvested for analysis. (A-B) Splenocytes were stained with anti-CD8, anti-Thy1.2, and anti-IFN-γ intracellularly. (A) Percentages of IFN-γ-producing CD8⁺ T cells among each respective group are indicated. Plots are gated on CD8⁺ T cells. (B) The mean absolute numbers ± SD of IFN-γ-producing cells per spleen are indicated (n = 4 per group). (C-D) Splenocytes were stained with anti-CD8, anti-Thy1.2, anti-CD62L, and Annexin V. (C) Percentage of Annexin V⁺ cells among transferred CD62L⁺ cells are indicated. Naïve plot is gated on CD62L⁺ cells. WT and IL-21R⁻ plots are gated on Thy1.2⁺CD8⁺CD62L⁺ cells. (D) The mean absolute numbers ± SD of Annexin V⁺CD62L⁺ cells among those transferred are indicated (n = 4 per group). Data are representative of three independent experiments.
4.2.3 Intrinsic IL-21 signaling is critical for the survival of activated CD8 T cells in vivo

Defective generation of VV-specific CD8 T cells in the absence of IL-21 signaling could be due to insufficient activation, proliferation, or effector differentiation, in addition to poor survival. To address these possibilities, naïve WT or IL-21R−/− Clone 4 HA-specific CD8 T cells (Thy1.2+) were transferred into congenic B10.D2 (Thy1.1+) mice that were subsequently infected with rVV-HA. 7 days later, splenocytes were analyzed for clonal expansion and effector differentiation of the Clone 4 CD8 T cells. Substantial clonal expansion and effector differentiation as measured by IFN-γ production were observed for WT Clone 4 T cells (Fig. 11A, B). This expansion was significantly (p <0.01) reduced in IL-21R−/− Clone 4 T cells (Fig. 11A, B). A similar degree of reduction was found in other lymphoid and non-lymphoid organs, including peripheral lymph nodes (PLN), liver, and lung (Fig. 11B). These data further support the observation with the polyclonal CD8 T cells (Fig. 10) and indicate that intrinsic IL-21 signaling is critical for clonal expansion of CD8 T cells in response to VV infection in vivo.

To address what contributes to the defective clonal expansion of IL-21R−/− CD8 T cells, we transferred naïve WT or IL-21R−/− Clone 4 CD8 T cells into naïve mice and subsequently infected the hosts with rVV-HA. 24 h after infection, both WT and IL-21R−/− Clone 4 CD8 T cells displayed a similarly activated CD44highCD69high phenotype compared to the naïve CD44lowCD69low phenotype (Fig. 12A), suggesting early activation of CD8 T cells is not affected by lack of IL-21 signaling. 3 days post-infection, CFSE
labeled WT and IL-21R−/− Clone 4 CD8 T cells underwent similar levels of proliferation as measured by CFSE dilution (Fig. 12B), suggesting that lack of IL-21 signaling does not affect CD8 T cell proliferation *in vivo*. Additionally, despite having a reduced clonal population, effector differentiation of IL-21R−/− Clone 4 CD8 T cells is not affected, since the IFN-γ production on a per cell basis is not affected based on the mean fluorescence intensity (MFI) of staining (Fig. 11A). Similarly, the production of TNF-α appears to be similar in the absence of IL-21 signaling (Fig. 12C). The phenotype of effector CD8 T cells as measured by CD62L downregulation and CD122 upregulation is also unchanged in IL-21R−/− Clone 4 CD8 T cells as compared to WT (Fig. 12C).

We next sought to determine whether the observed reduction in clonal population size seen in IL-21R−/− CD8 T cells was due to enhanced apoptosis in the absence of IL-21 signaling. We transferred naïve WT or IL-21R−/− Clone 4 CD8 T cells into naïve hosts that were subsequently infected with rVV-HA. 7 days later, splenocytes were stained with Annexin V to detect cells undergoing apoptosis. Similar to our finding with the polyclonal CD8 T cells (Fig. 10C, D), IL-21R−/− Clone 4 CD8 T cells had a significant increase in Annexin V positivity (41.3%) compared to WT Clone 4 cells (17.9%)(Fig. 12C). Taken together, these results suggest that although the activation, proliferation and effector differentiation of CD8 T cells in response to VV infection is unaffected by a lack of IL-21 signaling, the survival of activated CD8 T cells is critically dependent on intrinsic IL-21 signaling *in vivo*. 
Figure 11: CD8 T cell clonal expansion in response to VV infection is compromised in the absence of IL-21 signaling.
Figure 11: CD8 T cell clonal expansion in response to VV infection is compromised in the absence of IL-21 signaling. 1 x 10^4 purified naïve Clone 4 CD8 T cells (Thy1.2+) from either WT or IL-21R⁻ mice were adoptively transferred into congenic B10.D2 mice (Thy1.1+) that were subsequently infected with rVV-HA (5 x 10^5 PFU, i.p.). 7 days later, spleen and other lymphoid and nonlymphoid organs were harvested for analysis of expansion and function of clonotypic CD8 T cells. (A) Splenocytes were stained with anti-CD8 and anti-Thy1.2, and anti-IFN-γ intracellularly. The percentage of total clonotypic CD8 T cells among total lymphocytes is indicated (left panels); the percentage of IFN-γ-producing clonotypic cells among total CD8 T cells is indicated with the numbers in parentheses showing the MFI (x 10^3) of IFN-γ-producing clonotypic CD8 T cells (right panels). (B) The mean absolute numbers ± SD of clonotypic T cells per spleen, combined six peripheral lymph nodes (PLN), whole liver, or whole lung are indicated (n = 4 per group). Data shown are representative of four independent experiments.
Figure 12: IL-21 signaling is required for the survival, but not the activation or proliferation, of CD8 T cells. (A-B) 1 x 10^6 purified naïve Clone 4 CD8 T cells from WT or IL-21R^-/- mice were transferred to congenic recipients that were subsequently infected with rVV-HA (5 x 10^6 PFU, i.p.). Some mice were left uninfected (Naive). (A) 24 h post-infection, splenocytes were harvested and stained with antibodies to CD8, Thy1.2, and the activation markers CD44 or CD69. Percentages of CD44^high and CD69^high are indicated. (B) 3 days post-infection, in vivo division of CFSE-labeled clonotypic cells in the spleen was analyzed. (C) 1 x 10^4 purified naïve Clone 4 CD8 T cells from either WT or IL-21R^-/- mice was transferred into recipients that were subsequently infected with rVV-HA (5 x 10^5 PFU, i.p.). 7 days later, splenocytes were stained with anti-CD8 and anti-Thy1.2 and analyzed for the expression of surface markers and the production of the effector molecules. The percentages of TNF-α-producing, CD62L^low, CD122^high and Annexin V^+ clonotypic CD8 T cells are indicated. All plots are gated on CD8^+Thy1.2^+ cells. Data shown are representative of four independent experiments.
4.2.4 Intrinsic IL-21 signaling is required for the formation of memory CD8 T cells in vivo

The observation that the activated IL-21R−/− CD8 T cells survived poorly prompted us to study their ability to develop into memory cells. 42 days after infection, mice were harvested for analysis of memory cell formation in lymphoid and non-lymphoid organs. Consistent with our previous studies utilizing rVV-HA infection [70, 136, 148, 172], WT Clone 4 effector CD8 T cells had undergone contraction to form a memory pool, whereas IL-21R−/− Clone 4 effector CD8 T cells could not survive the contraction phase to develop into memory cells (Figs. 11 and 13A, B). To ensure that the lack of memory formation from IL-21R−/− Clone 4 T cells was not due to a low level below the threshold of detection in our system, we boosted mice at day 42 with recombinant adenovirus encoding HA (Ad-HA) to assess the recall response. We observed a robust recall expansion of WT Clone 4 memory CD8 T cells (Fig. 13C, D). However, there were still no detectable IL-21R−/−Clone 4 CD8 T cells (Fig. 13C, D). Collectively, our data indicate that intrinsic IL-21 signaling in CD8 T cells is required for the formation of long-lived memory cells in response to VV infection.
Figure 13: Defective CD8 memory formation in the absence of IL-21 signaling.
**Figure 13: Defective CD8 memory formation in the absence of IL-21 signaling.**

1 x 10^4 naïve Clone 4 CD8 T cells from either WT or IL-21R−/− mice was transferred into recipients that were subsequently infected with rVV-HA (5 x 10^5 PFU, i.p.). (A-B) 42 days later, spleen and other lymphoid and non-lymphoid organs were harvested for analysis of clonotypic CD8 T cells. (A) Splenocytes were stained with anti-CD8, anti-Thy1.2, and anti-IFN-γ intracellularly. The percentage of total clonotypic CD8 T cells among total lymphocytes is indicated (left panels); the percentage of IFN-γ-producing clonotypic cells among total CD8 T cells is indicated (right panels). (B) The mean absolute numbers ± SD of clonotypic T cells per spleen, combined six peripheral lymph nodes (PLN), whole liver, or whole lung are indicated (n = 4 per group). (C-D) 42 days post-infection with rVV-HA, mice were boosted with Ad-HA, and 5 days post-boost infection, lymphocytes were analyzed for clonotypic CD8 T cells. (C) The percentage of total clonotypic CD8 T cells among total splenic lymphocytes is indicated (left panels); the percentage of IFN-γ-producing clonotypic cells among total splenic CD8 T cells is indicated (right panels). (D) The mean absolute numbers ± SD of clonotypic T cells per spleen, combined six peripheral lymph nodes (PLN), whole liver, or whole lung are indicated (n = 4 per group). Data shown are representative of four independent experiments.
4.2.5 IL-21 enhances the survival of activated CD8 T cells via the STAT1 and STAT3 pathways

How does IL-21 promote CD8 T cell survival? It has been shown that IL-21 can activate the STAT1, STAT3, and STAT5 pathways [173, 174]. Furthermore, previous studies in our lab have described a requirement for STAT1 signaling in the survival of activated CD8 T cells in vivo [70]. To study whether these STAT pathways play a role in IL-21-mediated CD8 T cell survival, naïve polyclonal CD8 T cells were cultured in the presence of recombinant IL-21 and then examined for the activation of STAT1, STAT3 and STAT5 by intracellular staining for phosphorylated STAT1 (pSTAT1), pSTAT3, and pSTAT5. Marked activation of both STAT1 and STAT3, but not STAT5, was observed in cultures supplemented with IL-21, compared to the medium only control (Fig. 14A). To further determine whether the STAT1 and STAT3 pathways were required for IL-21-dependent CD8 T cell survival, naïve WT or STAT1<sup>−/−</sup> polyclonal CD8 T cells were retrovirally transduced with either empty vector or a STAT3 dominant negative (STAT3-D) vector and cultured in the presence of IL-21. We observed that CD8 T cell apoptosis was markedly reduced when WT cells with empty vector were cultured in the presence of IL-21, compared to WT cells with empty vector alone (33.4% from 81.4%). However, this inhibition of apoptosis by IL-21 was abrogated in the absence of STAT1, in the presence of STAT3-D, or both (Fig. 14B). We further observed that increased expression of the pro-survival factors Bcl-2 and Bcl-xL observed with addition of IL-21 to the culture, was also reduced when STAT1, STAT3, or both are blocked (Figure 14C).
Expression of the pro-apoptotic factor Bim was unchanged in these conditions (data not shown). Collectively, these data suggest that IL-21-dependent enhancement of CD8 T cell survival is mediated by both the STAT1 and STAT3 pathways and subsequent upregulation of Bcl-2 and Bcl-xL.
Figure 14: IL-21 activates the STAT1 and STAT3 signaling pathways, leading to the enhanced survival of CD8 T cells in vitro.
Figure 14: IL-21 activates the STAT1 and STAT3 signaling pathways, leading to the enhanced survival of CD8 T cells *in vitro*. (A) Purified polyclonal CD8 cells were cultured for 1 h in medium alone or medium supplemented with recombinant murine IL-21. Cells were stained with anti-CD8 and anti-pSTAT1, anti-pSTAT3, or anti-pSTAT5 intracellularly. (B-C) Purified polyclonal CD8 cells from WT or STAT1−/− mice were retrovirally infected with empty vector or STAT3-D and stimulated with soluble anti-CD3 and anti-CD28 in medium alone or medium supplemented with IL-21. (B) 4 days later, cells were stained with anti-CD8 and Annexin V. Percentage of Annexin V+ CD8 cells is indicated. Plots are gated on GFP+ cells. (C) GFP+ cells were sorted and mRNA was extracted and quantitative PCR was used to measure the expression of Bcl-2 and Bcl-xL. mRNA abundance was normalized to β-actin. Data are representative of two independent experiments. * = p≤.001.
4.2.6 IL-21 signaling on CD8 T cells promotes activation of STAT1 and STAT3 and induction of Bcl-xL in response to VV infection *in vivo*

We next examined whether IL-21 signaling on CD8 T cells promotes activation of STAT1 and STAT3 and expression of Bcl-xL upon VV infection *in vivo*. Naïve WT or IL-21R<sup>−/−</sup> Clone 4 CD8 T cells were transferred into congenic B10.D2 mice that were subsequently infected with rVV-HA. 5 days post-infection, splenocytes were harvested and stained intracellularly for pSTAT1 and pSTAT3. Significant activation of both STAT1 and STAT3 was observed in WT Clone 4 CD8 T cells upon VV infection *in vivo*, compared to the naïve control (Fig. 15A). However, the extent of STAT1 and STAT3 activation was reduced in IL-21R<sup>−/−</sup> Clone 4 T cells, compared to their WT counterparts (Fig. 15A). Similar to our *in vitro* results, this decrease in pSTAT1 and pSTAT3 activity was correlated with a decrease in the Bcl-xL level as measured by MFI in IL-21R<sup>−/−</sup> Clone 4 T cells (Fig. 15B). These results indicate that intrinsic IL-21 signaling in CD8 T cells is required for efficient activation of STAT1 and STAT3 as well as upregulation of Bcl-xL upon VV infection *in vivo*. 


Figure 15: IL-21 signaling promotes activation of the STAT1 and STAT3 pathways and upregulation of Bcl-xL in vivo. 1 x 10^4 purified naïve Clone 4 CD8 T cells (Thy1.2+) from either WT or IL-21R−/− were adoptively transferred into congeneric B10.D2 mice (Thy1.1+) that were subsequently infected with rVV-HA (5 x 10^5 PFU, i.p.), or left uninfected (Naïve). (A) 5 days post-infection, splenocytes were stimulated with PMA and Ionomycin and then stained with anti-CD8, anti-Thy1.2, as well as anti-pSTAT1, anti-pSTAT3, or an isotype control intracellularly. Percentage of pSTAT1+ and pSTAT3+ among clonotypic CD8 T cells is indicated. (B) 7 days post-infection, splenocytes were stained with anti-CD8, anti-Thy1.2, and anti-Bcl-xL intracellularly. MFI of clonotypic CD8 T cells is indicated. Plots are gated on CD8+Thy1.2+ cells. Data are representative of three independent experiments.
4.3 Discussion

Here, we demonstrate that CD4 T cell help for the CD8 T cell response is mediated by IL-21 and that direct action of IL-21 on CD8 T cells is critical for VV-specific CD8 T cell response in vivo. We further reveal that this cell-intrinsic IL-21 signaling is critical for the survival of activated CD8 T cells and CD8 T cells deficient in IL-21 signaling fail to develop into long-lived memory cells in response to VV infection in vivo. We further demonstrate that IL-21 promotes CD8 T cell survival by inducing the pro-survival molecules Bcl-2 and Bcl-xL in a STAT1- and STAT3-dependent manner in vitro and that CD8 T cells defective for IL-21 signaling had reduced levels of STAT1 and STAT3 activation and Bcl-xL upregulation in response to VV infection in vivo.

Recent studies have revealed that IL-21 is a key component of CD4 T cell help that is required for maintaining the CD8 T cell response during chronic LCMV infections [117-119]. This is achieved by direct action of IL-21 on virus-specific CD8 T cells to avoid deletion and thus, sustain immunity. Interestingly, IL-21 signaling is not required for the CD8 T cell response to acute LCMV infections [117-119]. This could be attributed to the observation that the primary CD8 T cell response during acute LCMV infection is independent of CD4 T cell help [97, 120]. In a model of CD4 T cell help-dependent CD8 T cell response to acute VV infection [148], here we show that IL-21 signaling is also essential for the primary CD8 T cell responses in vivo. Furthermore, we also provide direct evident that CD4 T cell help for the CD8 T cell response is mediated by IL-21. The
mechanisms underlying differential requirements for IL-21 in the CD8 T cell response to different pathogens remain elusive. It might be that some acute infections could induce factors that may compensate for the loss of IL-21 signaling. Thus, future studies are needed to address this question.

A previous study has shown that IL-21 in combination with IL-7 or IL-15 augments the proliferation of resting CD8 T cells in vitro in the absence of TCR signals; and promotes antigen-specific CD8 T cell expansion and their function in vivo in response to VV encoding HIV gp160 antigen [116]. Our data that direct IL-21 signaling on CD8 T cells is critical for the expansion of VV-specific CD8 T cells in vivo is in line with this observation. Furthermore, our results with clonotypic HA-specific T cells demonstrate for the first time that the defective clonal expansion and function of antigen-specific CD8 T cells in the absence of IL-21 signaling is due to poor survival, but not initial activation, proliferation or effector differentiation, of the activated antigen-specific CD8 T cells. Although IL-21 promotes resting CD8 T cell proliferation in vitro in the absence of TCR signals, CD8 T cell proliferation in vivo in response to VV infection is not affected by lack of IL-21 signaling, which could be due to TCR ligation and CD28 co-stimulation also inducing CD8 T cell proliferation in vivo.

The signals that promote the generation and maintenance of the memory T cell population remain incompletely defined. Studies have shown that the nature and the strength of TCR signals [136, 175, 176], the co-stimulation [177], as well as the
inflammatory milieu [178], can influence the formation of memory T cells. In addition, the common-γ chain cytokines IL-7 and IL-15 play an important role in the maintenance of memory CD8 T cells by promoting homeostatic expansion [7, 179]. Furthermore, CD4 T cell help is critical for the generation of long-lived, functional memory CD8 T cells [98, 120, 121]. Here we provide evidence that CD4 help is mediated by another common-γ chain cytokine, IL-21, and that CD8 T cells defective for IL-21 signaling could not survive the contraction phase to develop into long-lived memory cells in response to VV infection in vivo. The observation that lack of memory CD8 T cell formation in the absence of IL-21 signaling, even after the secondary challenge, is somewhat different from that seen in CD4−/− mice where the CD8 memory pool is detectable albeit significantly reduced [148]. This difference could be due to low levels of IL-21 produced by non-CD4 T cells such as NKT cells in CD4−/− mice [111].

How does IL-21 signaling in CD8 T cells promote their survival? Previous studies have shown that IL-21 can activate the STAT1, STAT3, and STAT5 pathways [173, 174]. In CD8 T cells, IL-21 signaling can preferentially activate the STAT1 and STAT3 pathways, leading to enhanced T cell proliferation in combination with IL-15 in vitro in the absence of TCR signals [174]. In addition, it has been shown that STAT1 signaling in CD8 T cells is required for CD8 T cell survival and memory formation in vivo [70]. Here we show that IL-21 signaling activates both STAT1 and STAT3 pathways in vitro and in vivo, and that IL-21 enhances the survival of anti-CD3 activated CD8 T
cells in a STAT1 and STAT3-dependent manner. We further demonstrate that IL-21 signaling upregulates the expression of the pro-survival molecules Bcl-2 and Bcl-xL in a STAT1- and STAT3-dependent pathway in activated CD8 T cells. Collectively, our data support a model that IL-21 signaling promotes the survival of activated CD8 T cells by inducing pro-survival molecules, such as Bcl-2 and Bcl-xL, in a STAT1 and STAT3-dependent fashion. Indeed, it has been shown in other immune cells that activation of STAT1 or STAT3 can upregulate Bcl-2 and Bcl-xL, leading to cell survival [180-182].

Down-regulation of TRAIL expression by CD4 T cell help has been shown to protect memory CD8 T cells from activation-induced cell death during a recall expansion [125]. A more recent study has suggested that CD4 T cell help consists of both TRAIL-dependent and –independent mechanisms [160]. Indeed, we have previously shown that in the absence of CD4 T cell help, TRAIL expression is up-regulated in the activated CD8 T cells, whereas Bcl-XL expression is down-regulated [148], suggesting that both the TRAIL pathway and the intrinsic apoptotic pathway may be involved in promoting the survival of activated CD8 T cells. Thus, we cannot rule out the possibility that IL-21-STAT1/STAT3 signaling may also result in downregulation of TRAIL, leading to reduced apoptosis of activated CD8 T cells. Thus, future studies will be needed to define whether TRAIL is involved in IL-21-dependent CD8 T cell survival.

In conclusion, we have shown that CD4 T cell help for CD8 T cell response to VV infection is mediated by IL-21. This is achieved by direct action of IL-21 on CD8 T cells to
promote their survival via a mechanism dependent on activation of the STAT1 and STAT3 pathways and subsequent upregulation of Bcl-2 and Bcl-xL. Furthermore, effector CD8 T cells do not survive the contraction phase to differentiate into long-lived memory cells in the absence of intrinsic IL-21 signaling. These results identify a critical role for direct IL-21 signaling in CD8 T cell survival and memory formation following an acute viral infection in vivo and may have important implications for the design of effective strategies for treating infectious diseases and cancer.

5. Overall Conclusions and Future Directions

As the essential arm of the immune system necessary for protection against a variety of viral and bacterial pathogens, the adaptive CD8 T cell response remains a key area of research for the optimization and manipulation of different vaccination strategies. In particular, vaccinia virus possesses strong therapeutic possibilities due to its success as a vaccine and its ability to be engineered to express foreign antigens to stimulate an immune response. To that end, studying the CD8 T cell response to VV presents a useful tool for future vaccine design.

The goal of this thesis was to determine if and how CD4 T cells augment the CD8 T cell response to VV infection. Using a model of HA-specific transgenic CD8 T cells and i.p. infection with rVV-HA, we demonstrated that CD4 T cells are not required for the activation and effector differentiation of CD8 T cells during the primary response. This is
consistent with earlier observations documenting that pathogens can activate DCs directly and bypass the need for CD4 T cell help [72]. Although they are dispensable for CD8 T cell activation, CD4 T cells are required for the optimal survival of activated CD8 T cells and, in their absence, there is a reduction in the size of the CD8 effector pool. The effectors generated in the absence of CD4 T cell help are phenotypically indistinguishable from those generated in CD4-sufficient environments. This reduced size of the effector pool translates to a reduced, albeit relatively numerically stable, memory pool.

We next showed that the help provided by CD4 T cells is essential for fully functional recall responses of memory CD8 T cells by promoting their survival. Consistent with previous reports, memory CD8 cells generated in the absence of CD4 T cell help undergo significantly more cell death during the recall response. Furthermore, CD4 T cells are required during the recall response to promote CD8 survival. This suggests the requirement for CD4 help during the CD8 response is twofold: they are required for the survival of activated CD8 T cells both during the primary and recall responses and are also required for the future survival of memory CD8 cells during the recall response. These findings were validated by two other studies: one using a model of the endogenous CD8 T cell response to VV and the other employing a system of Listeria monocytogenes infection [100, 183].
How CD4 help programs memory CD8 T cells for more efficient survival during the recall response is still unclear. Although studies utilizing LCMV show no requirement for CD4 T cells in the generation of the CD8 effector pool, they do point to a role for CD4 T cells in the programming of efficient CD8 recall responses [98, 120]. This programming is thought to be due to the increased expression of TRAIL in CD8 T cells that do not receive CD4 help [125]. Consistent with these findings, we observed significant upregulation of TRAIL in effector CD8 T cells generated in CD4-deficient settings during VV infection. Whether this TRAIL upregulation is responsible for the enhanced cell death of “helpless” memory CD8 T cells during the recall response to VV infection remains to be determined.

We next sought to determine how CD4 T cells contribute to the survival of activated CD8 T cells during VV infection. We hypothesized that CD4 T cells could either indirectly provide survival signals by acting on an intermediate cell, likely DCs, or they could directly provide survival signals to activated CD8 cells. Using in vitro culture systems, we determined that CD4 T cells do not provide survival signals to CD8 T cells by acting on DCs or through cell-to-cell contact mechanisms. This suggests that CD4 T cells secrete a soluble factor to promote CD8 survival. Using real-time PCR, we measured the levels of mRNA for the common-γ-chain cytokines produced by naïve and activated CD4 T cells because, as described in Chapter 1, these common-γ-chain cytokines are the key regulators of the CD8 T cell response. Unsurprisingly, CD4 T cells
upregulate IL-2 production after activation. More surprising to us was that activated CD4 T cells also significantly upregulate IL-21. Upon further examination, we determined that the addition of IL-21 to cultures can enhance CD8 survival. We next determined that CD4 help for CD8 survival in vitro is mediated by IL-21.

In order to establish the in vivo relevance of IL-21 signaling in CD8 T cells, we transferred polyclonal CD8 T cells from either WT or IL-21R−/− mice and subsequently vaccinated with rVV-HA i.p.. Seven days later, CD8 T cells deficient in IL-21 signaling had a reduced effector pool and were undergoing significantly higher levels of apoptosis. We then used the transgenic HA-specific CD8 T cells to determine that, much like what we observed in the CD4-deficient setting, activation and effector differentiation is unchanged in the absence of IL-21 signaling. CD8 T cells defective for IL-21 signaling could not survive the contraction phase to develop into long-lived memory cells. This is somewhat different from observations in CD4-deficient mice, where the CD8 memory pool is detectable but significantly reduced. One likely possibility for this difference is that IL-21 can also be produced by NKT cells in CD4−/− mice. Studies examining whether NKT cells can secrete IL-21 in response to VV infection will be necessary to validate this hypothesis.

We next wanted to determine the signaling pathways utilized by IL-21 to promote CD8 T cell survival. Since several other studies have shown that IL-21 can signal through the STAT1, STAT3, and STAT5 pathways, we assessed which of these
were activated in CD8 T cells stimulated with IL-21 [173, 174]. We observed that IL-21 activates the STAT1 and STAT3 pathways in vitro. Furthermore, both STAT1 and STAT3 pathways are required for IL-21-mediated survival in vitro, potentially by regulating the levels of the prosurvival molecules Bcl-2 and Bcl-xL. We confirmed our in vitro findings using HA-specific CD8 T cells and clearly saw that levels of phosphorylated STAT1 and STAT3 are reduced in the absence of IL-21 signaling, as is the protein level of Bcl-xL. Collectively, these data support a model where CD4 T cell help is mediated by IL-21 signaling, which promotes the STAT1- and STAT3-dependent upregulation of Bcl-2 and Bcl-xL.

This body of work has provided direct evidence that CD4 T cells promote the survival of activated CD8 T cells in response to VV infection by secreting high levels of IL-21. Still unclear however, is the role of IL-21 at other phases of the CD8 response. Based on the requirement for CD4 T cells during various stages of the CD8 response, it is likely that IL-21 will be indispensable during the recall response. Whether it is required at other phases of the CD8 response remains to be determined. Studies utilizing an estrogen-receptor-based Cre system to abrogate IL-21 signaling at different phases of the response would be necessary to elucidate the role(s) of IL-21 throughout the CD8 response.

Another important question centers upon uncovering the exact source of IL-21 during VV infection. While it is known that CD4 T cells and NKT cells can produce IL-
21, we still do not know which subset of CD4 T cells is the major producer during VV infection. Recent work has suggested that Th17 cells can produce high levels of IL-21 [169]. Studies have not determined a specific role for Th17 cells during VV infection; whether VV infection can induce the activation of a population of Th17 cells and how they may promote the CD8 response remains to be determined. Still, there is the possibility that a subpopulation of CD4 T cells, follicular-helper T cells which aid in the generation of antibody responses, are the source of IL-21 [164]. Future studies determining the source of IL-21 will lend insight into modulating IL-21 production in different settings.

Since NKT cells are the only other cell type that produces IL-21, our work using CD4-deficient mice suggests that NKT cells can become activated during VV infection and produce IL-21. To date, this is the first work suggesting that NKT cells can respond to VV infection in vivo. If NKT cells can respond to VV infection, it would be useful to determine whether this recognition is antigen-specific or whether the NKT cells are activated due to high levels of circulating proinflammatory cytokines, as high IL-12 and IFN signals can promote NKT cell activation [184, 185]. If NKT cell activation is antigen-specific, studies elucidating the epitope from vaccinia would need to be performed. Furthermore, since NKT cells are such strong manipulators of the immune response through robust cytokine production, they may play significant roles in regulating the CD8 T cell response [184, 185]. Identifying the potential requirement for NKT cell
activation to modulate the CD8 response would further the potential usefulness of this body of work for more efficient vaccine design.

Recent studies have elucidated a role for CD4-derived IL-21 in maintaining the effector pool during chronic LCMV infection [117-119]. Interestingly, IL-21 signaling was not required for the CD8 response to acute LCMV infection in these studies. It is critical to note that the primary response to acute LCMV infection has been shown to be independent of CD4 T cell help, suggesting that CD4-derived IL-21 should not be expected to play a role in that model [97, 120]. Since the primary response to VV infection is indeed dependent on CD4 T cell help, we propose that CD4 T cell help for CD8 survival is mediated by IL-21. Furthermore, the dependence on IL-21 may dictate the requirement for CD4 T cell help for different pathogen infections.

The mechanisms underlying the differential requirement for IL-21 in different pathogen settings are still unknown. It is possible that different pathogens illicit the production of different cytokines or other soluble factors to compensate for reduced IL-21 production. The STAT1 and STAT3 pathways can be activated by a variety of different soluble factors, such as growth factors (G-CSF and GM-CSF) and inflammatory cytokines (IL-6, TNF-α, and interferons) [186-190]. Whether these factors signal via the STAT1 and STAT3 pathways in CD8 T cells is still unclear. To this end, it has been shown that early IFN signaling is required for the survival of activated CD8 T cells in a STAT1-dependent signaling pathway [70]. It is possible that soluble factors induced by
vaccinia infection can induce significant levels of phosphorylated STAT1 and STAT3 to bypass the requirement of IL-21 signaling. Further experiments to elucidate whether inflammatory cytokines or other growth factors can compensate for IL-21 signaling will be required to test this hypothesis.

Another possibility is that different pathogens may interact with different receptors present on CD8 T cells to augment their survival. T cells express a number of the innate pattern recognition receptors (PRRs) that recognize a variety of pathogen-associated molecular patterns (PAMPs) [191-193]. Although these were initially described in terms of the innate immune system, recent developments on how signaling through these PRRs can augment a CD8 T cell response has demonstrated that CD8 T cells do indeed recognize PAMPs and this recognition is crucial to their overall function and survival [172, 194-196]. Our lab has previously demonstrated that the innate TLR2-MyD88 pathway is required for the survival of activated CD8 T cells in a PI3K-dependent signaling cascade during VV infection [172]. As different pathogens possess different ligands for the innate pattern recognition receptors found on cells of the immune system, it is possible that signaling through these receptors can override the requirement for IL-21 signaling for CD8 T cell survival. Future studies are needed to address these different possibilities and to determine what combination of pro- and anti-apoptotic pathways, both cytokine-dependent and -independent, are involved in generating optimal CD8 T cell responses to VV infection.
Collectively, this thesis has demonstrated a clear requirement for CD4 T cell help for a complete CD8 T cell response to VV infection. This CD4 help is achieved by the secretion of IL-21. Thereby, our work identifies one key component necessary for a sufficient response to vaccinia viral infection. These findings will be critical when generating more powerful and effective vaccination strategies for the treatment of infectious disease and cancer in a variety of immunocompromised patients.
References


103. Yang, Y., et al., Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant


106


Biography

I was born Patricia Lynne Novy on April 9, 1984 in Northridge, CA. I was the first child of Robert and Judith Novy, and I lived in spoiled bliss, playing with our dog Benson, until July 6, 1984 when my brother Chris entered the world. I graduated from Paraclete High School in 2001 and moved to Davis, CA for my first taste of college freedom.

In 2005, I graduated with honors from the University of California at Davis with a Bachelor’s of Science in Biological Sciences with an emphasis in Medical Microbiology. I moved to Durham, NC to pursue my Ph.D. in Immunology at Duke University. While in the laboratory of Dr. Yiping Yang, I published one article titled “CD4 T cells are required for CD8 T cell survival during both the primary and recall responses” in the Journal of Immunology in 2007. Another article titled “Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection” is currently under review. I attended the Keystone Conference on NK and NKT cell biology in February 2008 and was awarded the Duke University Travel Fellowship. In May 2010, I attended the AAI Immunology 2010 conference in Baltimore and was invited to give an oral presentation detailing my work in addition to a poster presentation. I also received the Duke University Travel Fellowship and the AAI Trainee Abstract Award. Since receiving my Ph.D, I have to decided to stay in Dr. Yang’s lab to continue research while exploring future career possibilities.