EBV-Specific CD4+ T Cell Responses in Acute Infectious Mononucleosis: a Dissertation

Melissa Lynn Precopio

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A Dissertation Presented

By

Melissa Lynn Precopio

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

DOCTOR OF PHILOSOPHY

April 1, 2004

Program in Immunology/Virology
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Experimental results presented in this thesis dissertation have appeared in the following publications:


EBV-SPECIFIC CD4⁺ T CELL RESPONSES IN ACUTE INFECTIOUS MONONUCLEOSIS

A Dissertation Presented

By

Melissa Lynn Precopio

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April 1, 2004
DEDICATION

This thesis is dedicated to

My parents
Edward and Linda Precopio

Your unwavering support and unconditional love
have meant the world to me

Thank you, I love you both
ACKNOWLEDGEMENTS

I first and foremost thank my mentor, Katherine Luzuriaga, M.D. for her guidance, support, patience, and positive attitude. I am very grateful that I had the opportunity to pursue my research interests in her lab and appreciate the direction and encouragement I have received from her over the years. Her confidence and enthusiasm for science have been a great example for me, and I am very thankful for all that I have learned as her student.

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scientific and personal relationship that we developed while we were members of the lab together. I also thank Melissa Farrow and Siwei Nie for their support and friendship and wish them the best.

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ABSTRACT

Epstein-Barr virus (EBV) is a ubiquitous herpesvirus that establishes a lifelong latent infection of B cells. It is usually asymptomatic in healthy individuals; however, individuals with compromised immunity often develop EBV-induced lymphoma. EBV also encodes potential oncogenes that can contribute to tumorigenesis. Therefore, vaccine and immunotherapeutic strategies targeting EBV are desirable. Recent studies have shown that infusion of EBV-specific CD8+ T cells can elicit remission of lymphomas arising after administration of immunosuppressive drugs during transplantation, suggesting an important role for T cells in the prevention of EBV-induced malignancy. A better understanding of the cellular immune components involved in the control of EBV will aid in the development of methods to prevent infection and/or treat EBV-associated disease.

While EBV infection is usually acquired asymptotically during childhood, primary infection of adolescents and young adults can result in an illness termed acute infectious mononucleosis (AIM). Because of the characteristic symptoms of the illness, individuals with AIM can be readily identified and diagnosed with acute EBV infection. Thus, primary CD4+ and CD8+ T cell responses against the virus can be evaluated. It has been previously found that there is a marked expansion of lytic EBV protein-specific CD8+ T cells early during AIM, with delayed detection of lower frequencies of latent EBV
protein-specific CD8$^+$ T cells. The magnitude and specificity of CD4$^+$ T cell responses during AIM has been less well characterized.

This thesis dissertation presents data from both functional assays and direct staining experiments documenting the timing, magnitude, and antigen-specificity of CD4$^+$ T cells over the course of primary EBV infection. Lytic and latent protein-specific CD4$^+$ T cells were readily detected by intracellular IFN-γ production at presentation with AIM and declined rapidly thereafter. Blood EBV load was also quantitated and found to decrease over time following AIM. By contrast, CD8$^+$ T cell IFN-γ responses remained high for several weeks following presentation with AIM.

Direct staining of lytic epitope-specific CD4$^+$ T cells during AIM revealed high frequencies of virus-specific cells with low proliferative and IFN-γ-producing potential. Blood EBV load in these patients was persistently high through 6 wk following AIM. These data suggest a relationship between high EBV load during acute infection and impaired EBV-specific CD4$^+$ T cell responses, which are compatible with impaired CD4+ T cell responses reported during high viremia associated with other viral infections. This may represent a mechanism by which persistent viruses, such as EBV, are able to establish a life-long infection in their hosts.
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# ABBREVIATIONS

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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AIM</td>
<td>Acute infectious mononucleosis</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>β2m</td>
<td>Beta-2 microglobulin</td>
</tr>
<tr>
<td>BLCL</td>
<td>B lymphoblastoid cell line</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CT</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>E</td>
<td>Early</td>
</tr>
<tr>
<td>EBNA</td>
<td>EBV-associated nuclear antigen</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>L</td>
<td>Late</td>
</tr>
<tr>
<td>Lytic-specific CD4+ T cell</td>
<td>CD4+ T cell specific for an EBV lytic protein</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic cell sorting</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MHV</td>
<td>Murine gammaherpesvirus</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PTLD</td>
<td>Post-transplant lymphoproliferative disease</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
</tr>
<tr>
<td>XLP</td>
<td>X-linked lymphoproliferative disease</td>
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</table>
CHAPTER I

INTRODUCTION

A. Family Herpesviridae

*Herpesviridae* consists of a group of ubiquitous viruses that infect and establish lifelong persistence in their hosts. Classification within the family *Herpesviridae* is based on the following structural properties: a large, double-stranded linear DNA genome enclosed within an icosahedral capsid, a lipid envelope with glycoprotein spikes, and tegument, or space between the nucleocapsid and the outer lipid membrane. Herpesviruses encode numerous gene products that have many functions, including gene regulation, viral replication, and immune modulation. The virus replication cycle results in lysis of the infected cell. A common characteristic of all herpesviruses is their ability to establish latency in a specific cell type or subset of cells. During latent infection, the viral genome circularizes and is transcriptionally restricted. Thus, herpesviruses are never cleared, but are carried in their hosts for life. Latency is clinically asymptomatic; however, viral reactivations can result in various clinical manifestations.

Herpesviruses are prevalent throughout nature and have been isolated from most animal species. They are divided into three subfamilies (alpha, beta and gamma) based mainly on host range and length of reproductive cycle. Human alphaherpesviruses include herpes simplex-type 1 (HSV-1), which
causes cold sores, herpes simplex-type 2 (HSV-2), or genital herpes, and varicella/zoster (VZV), which causes chicken pox during primary infection. Human betaherpesviruses include cytomegalovirus (CMV) and roseola virus (HHV-7). Members of the human gammaherpesvirus family include Epstein-Barr (EBV) and Kaposi's sarcoma (KSHV).

B. Epstein-Barr Virus (EBV)

1. Brief history and epidemiology

In the mid-1960's, herpesvirus-like particles were visualized by electron microscopy from Burkitt's lymphoma-derived lymphoid cells (1, 2). The virus, later named Epstein-Barr virus (EBV), was soon thereafter found in other lymphoid tumor cells. Because transformed lymphocytes could be isolated from the blood of acute infectious mononucleosis (AIM) patients (3), oropharyngeal secretions from AIM patients were tested and found to be able to transform lymphocytes (4). It was later determined that EBV was indeed the causative agent of AIM (5, 6). But surprisingly, it was also found that over 90% of the general population had been infected with the virus, as evidenced by serum antibodies reactive with EBV (7).

EBV is mainly transmitted through saliva. Primary EBV infection usually occurs during childhood and is most often asymptomatic (8). However, when primary infection is delayed until adolescence or later, which is most common in the developed Western world, presentation with symptoms of AIM can result (9).
After resolution of acute infection, the virus persists for life, rarely causing any clinical consequence to the host. However, due to its transforming ability in vitro, it is not surprising that EBV has been associated with many malignancies. These include Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease. Ongoing cellular immunity is crucial for the control of EBV. Post-transplant lymphoproliferative disease (PTLD) often results when immunosuppressive drugs are administered during transplantation. Additionally, acute EBV infection can cause a fulminant infectious mononucleosis, resulting in death of individuals with a genetic defect in SLAM-associated protein (SAP) (10-12). This gene is carried on the X chromosome, and thus the disease is known as X-linked lymphoproliferative disease (XLP).

2. Viral life cycle

2.1 Latent infection

The primary target of EBV infection is the B lymphocyte. Viral entry is mediated by binding of the viral glycoprotein, gp350/220 to its receptor, CD21, on the B cell, followed by vesicular internalization of the virus (13, 14). While the early events after viral fusion and internalization are not fully understood, circularization of the viral genome into an episome within the nucleus is known to occur by 16 h after infection (15, 16). In vitro infection of B cells invariably results in activation, proliferation, and transformation of the cells into continuously growing B lymphoblastoid cell lines (BLCLs). This is the result of a complex viral
gene transcription program that drives B cell activation and proliferation. The key transcriptional activator for this program is EBNA-2, which is upregulated to consistent levels by 24 to 32 hours post-infection (15).

EBNA-2 transactivates both cellular genes involved in B cell activation as well as the expression of viral latent genes by transcriptionally transactivating all of the latent gene promoters. The latent genes expressed by proliferating BLCLs include 6 nuclear proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP), 3 membrane proteins (LMP-1, -2A, and -2B), and 2 virally encoded RNAs (EBER-1 and -2). 

In vitro transformation of B cells requires EBNA-2 and LMP-1, but EBNA-LP, -3A, and -3C also play a crucial role in transformation (17). EBNA-1 is required for maintenance of the episomal viral genome in infected cells.

A key difference between in vitro and in vivo EBV infection of B cells is that EBV rarely causes outgrowth of transformed B cells in its host, but instead persists quiescently in resting memory B cells (18). The model for EBV infection and persistence in vivo, proposed by David A. Thorley-Lawson and colleagues, maintains that EBV manipulates normal B cell biology to gain access to the memory B cell compartment (Figure 1.1). It does so mainly through the actions of LMP-1 and -2a, which are constitutively active homologs of CD40 and the B cell receptor (BCR), respectively (19, 20). Thus, infected B cells receive activation and rescue signals analogous to antigen activation. While these signals result in continuous cell growth in vitro, B cells are able to differentiate out of cell cycle into resting memory cells in vivo, enabling persistence of the virus in
Figure 1.1: Model of the proposed parallels between EBV and antigen driven B cell activation and differentiation (from Thorley-Lawson, D.A. and Babcock, G.J. 1999 Life Sciences 65:1433-1453). LMP-1 and -2 are functional homologs of CD40 and BCR, respectively, and drive activation and differentiation of infected B cells. The ultimate result is the persistence of EBV in resting memory B cells. EBV-associated lymphomas with patterns of EBV gene expression corresponding to the stages of EBV-induced differentiation are shown at the bottom.
a cell type with limited gene expression and therefore less sensitive to immunosurveillance by T cells. A schematic diagram of the life cycle of primary and persistent EBV infection in its host is shown in Figure 1.2.

2.2 Lytic infection

As in vitro cell systems fully permissive for lytic EBV infection have yet to be discovered, the lytic cycle of infection can only be studied by inducing latently infected cells into lytic replication. The lytic cycle follows the sequential expression of immediate early (IE), early (E) and late (L) viral genes, resulting in lysis of the infected cell and release of progeny virions. The principal known IE genes are BZLF-1 and BRLF-1, which function as viral transcription factors (17). The E genes, which are defined by their transcription in the absence of viral DNA synthesis, function in viral transcription, viral replication, and cell survival (17). The viral L genes are mainly structural and involved in virion formation. One non-structural L gene is BCRF-1, which is a homolog of the human IL-10 gene and likely functions in immunomodulation (21).

The primary cell type(s) permissive for lytic EBV infection in vivo is unknown, but epithelial cells of the oropharynx are generally thought to be targets of lytic infection. This issue is controversial, however, as in situ hybridization studies have detected viral transcripts in desquamating epithelial cells and resident B cells of the tonsillar epithelium (22). Evidence for EBV
Figure 1.2

<table>
<thead>
<tr>
<th>Primary Infection</th>
<th>Persistent Infection</th>
</tr>
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<tbody>
<tr>
<td><strong>Saliva</strong></td>
<td><strong>EBV</strong></td>
</tr>
<tr>
<td><strong>Oropharynx</strong></td>
<td><strong>EBV</strong></td>
</tr>
<tr>
<td><strong>Epithelium</strong></td>
<td><strong>EBV</strong></td>
</tr>
<tr>
<td><strong>Lymphoid tissue and peripheral</strong></td>
<td><strong>EBV</strong></td>
</tr>
<tr>
<td><strong>Blood</strong></td>
<td><strong>EBV</strong></td>
</tr>
<tr>
<td>Lytic EBV-infected B cell</td>
<td>LMP-1</td>
</tr>
<tr>
<td>EBV-infected B cell</td>
<td>LMP-2</td>
</tr>
<tr>
<td>Latently infected, resting memory</td>
<td>LMP-1</td>
</tr>
<tr>
<td>B cells</td>
<td>LMP-2</td>
</tr>
<tr>
<td>Cytotoxic T cell</td>
<td>Reactivated</td>
</tr>
<tr>
<td>Natural killer cell</td>
<td>EBV-infected B cell</td>
</tr>
<tr>
<td><strong>Resting B cell</strong></td>
<td><strong>EBV-infected B cell</strong></td>
</tr>
</tbody>
</table>
EBV is transmitted through saliva and infects cells of the oropharynx. It can then initiate productive lytic infection or its latent program of infection. Infected cells that express EBV proteins can be targets of host T cell responses; resting memory B cells that do not express viral proteins evade host responses. Periodic reactivation results in production and secretion of new virions into the saliva.
infection of epithelial cells comes from the detection of EBV genome in the epithelial cells of nasopharyngeal carcinomas (23).

3. EBV-associated malignancies

The oncogenic potential of EBV is evident in its ability to transform B cells. In fact, EBV has been associated with an increasing number of lymphomas and epithelial malignancies. While the role of EBV in some malignancies is well-defined, the association of EBV with other tumors is unclear. B cell lymphomas arising in immunocompromised hosts, such as XLP, PTLD, and AIDS patients, constitute the most apparent role for EBV in tumor pathogenesis (22). EBV is also frequently detected in Burkitt’s lymphoma, Hodgkin’s disease, and nasopharyngeal carcinoma. For these malignancies, EBV appears to act along with other cofactors to cause disease.

EBV-associated diseases are categorized by the pattern of EBV gene expression found in the tumors (Figure 1.1). All tumors express EBNA-1, and Burkitt’s lymphomas (type I latency) express only this protein. Latency types II and III express other latent proteins in addition to EBNA-1, with latency type III characterized by expression of all latent EBV proteins. Latent gene expression of tumors has important implications for immunotherapeutic strategies to eliminate EBV-associated malignancies.

C. Role of T cell immunity in the control of persistent viral infections
1. Murine models

Studies of viral immunity in mice have shown that T cells are key mediators of virus control and elimination (24). Virus-specific CD8\(^+\) T cells are able to kill virus-infected cells and secrete antiviral cytokines such as IFN-\(\gamma\). Virus-specific CD4\(^+\) T cells can mediate antiviral effects directly, through cytokine secretion, or indirectly by the provision of "help" to B cells and CD8\(^+\) T cells. After resolution of acute infection, virus-specific memory CD8\(^+\) and CD4\(^+\) T cells persist and, along with neutralizing antibody, prevent re-infection with the same virus.

T cell immunity to lymphocytic choriomeningitis virus (LCMV) in mice has been the subject of much investigation. As LCMV-specific CD8\(^+\) and CD4\(^+\) T cells expand, virus is cleared. Frequencies of LCMV-specific T cells then decline until the memory population is established. LCMV-specific CD8\(^+\) T cells expand to higher frequencies than LCMV-specific CD4\(^+\) T cells, but both T cell populations are retained into long-term memory.

Macrophage- and lymphocyte- tropic strains of LCMV can cause a chronic infection in mice, lasting up to several months (25). CD8\(^+\) T cells are an essential component of LCMV clearance, as \(\beta_2m^{-}\) mice are unable to clear either acute or chronic LCMV infection (26). However, CD4\(^+\) T cells also constitute an important component of the LCMV responses during chronic infection. While CD4-depleted mice are able to clear acute LCMV infection by the parental strain, CD4 depletion during the acute phase of chronic LCMV infection results in lifelong
viral persistence (26). Studies using CD4-deficient mice suggest that the inability of these mice to clear the virus is due to the deletion and functional unresponsiveness of LCMV-specific CD8$^+$ T cells (27).

Murine gammaherpesvirus (MHV)-68 also establishes a persistent infection in mice. Resolution of acute MHV-68 infection is mediated by CD8$^+$ T cells (28); however, virus titers and virus-infected B cells increase over time in CD4-deficient mice and result in death of the animals (29). Unlike chronic LCMV infection of CD4-deficient mice, MHV-68-specific CD8$^+$ T cells are not deleted or functionally unresponsive (30). Secretion of IFN-γ by CD4$^+$ T cells likely plays an important role in mediating control of persistent infection with MHV-68 (31).

2. Human viral infections

T cell immunity is thought to be important in the control of persistent infections caused by several human viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), CMV, and EBV. During the acute phase of HIV infection, the initial drop in viremia is temporally associated with the emergence of HIV-specific CD8$^+$ T cells (32). Sufficient immune pressure is exerted on the virus by CD8$^+$ T cells to result in sequence mutations allowing escape from CD8$^+$ T cell recognition (33). Depletion of CD4$^+$ T cells during acute and persistent HIV infection results in reduced ability to control both HIV and infection with opportunistic pathogens.
CD4+ T cell responses are believed to be particularly important for the control of HCV infection. Strong HCV-specific CD4+ and CD8+ T cells responses are detected during acute infection in individuals that clear the virus; these responses also coincide temporally with clearance of virus (34). Patients that develop a chronic infection with HCV have generally weaker and less broad HCV-specific CD4+ T cell responses than those able to clear the virus (35, 36).

Immunosuppressed individuals, such as transplant recipients and HIV-infected patients, often experience CMV- and EBV-associated diseases. This suggests that virus-specific T cell responses are very important in controlling persistent CMV and EBV infection. In fact, adoptively transferred CTL clones from allogeneic bone marrow donors can prevent CMV disease in transplant recipients (37). CMV-specific CD4+ T cells also appear to be important in preventing CMV disease in renal transplant recipients (38). EBV-induced PTLD has also been successfully treated and/or prevented by infusions of EBV-specific T cells (39).

Primary immune responses against acute CMV infection have been characterized in CMV-seronegative renal transplant recipients of seropositive organ donors. CMV-specific CD4+ T cells, detected by intracellular IFN-γ staining, peak at a median of 7 d after first detection of CMV DNA in the peripheral blood (40). Frequencies of CMV-specific CD4+ T cells then decline rapidly, while CMV DNA remains high for several weeks. The kinetics of CMV-specific CD4+ and CD8+ T cell responses were further analyzed in asymptomatic
vs. symptomatic transplant recipients (38). For both asymptomatic and symptomatic patients, detection of CMV DNA and CMV-specific CD8\(^+\) T cell responses were comparable, with a median of 26-27 d for detection of CMV DNA and 21-24 d thereafter for detection of CMV-specific CD8\(^+\) T cells. Antibody responses were also similar in asymptomatic and symptomatic patients. However, detection of CMV-specific CD4\(^+\) T cell responses is delayed in symptomatic patients, with a median detection time of 39 d (vs. 10 d for asymptomatic patients) after detection of CMV DNA. These data suggest that CMV-specific CD4\(^+\) T cell responses are important in preventing CMV disease in transplant recipients.

D. Acute EBV infection as a system for studying the generation and maintenance of human T cell responses

1. Acute Infectious Mononucleosis (AIM)

Approximately 50% of individuals that experience primary EBV infection during young adulthood present with clinical symptoms of AIM. These include fever, sore throat, fatigue, lymphadenopathy, and splenomegaly. AIM is clinically diagnosed by positive Monospot test and the presence of atypical lymphocytes, with the confirmation of acute EBV infection through detection of anti-EBV viral capsid antigen IgM. Disease symptoms likely result from the vigorous T cell response because anti-viral agents do not affect disease course (41), and symptoms can persist long after reduction of virus load. As AIM is a clear
indication of primary EBV infection, and there is a relatively high incidence of AIM in the adolescent population, it has provided a means for studying primary EBV-specific T cell responses.

Although symptoms of AIM indicate primary EBV infection, there is an incubation period between virus transmission and development of clinical symptoms, the duration of which is estimated to be between 4-6 weeks. This incubation period, which does not commonly occur after laboratory infections of mice, also occurs during other human virus infections and may reflect the inoculation dose of virus in a natural infection vs. an experimental one. It is important to note, then, that EBV-specific immune responses characterized during the symptomatic phase of infection may not accurately represent the earliest stages of the acute response.

2. *EBV-specific CD8+ T cells*

High frequencies of EBV-specific CD8+ T cells have been documented during AIM. Although lytic and latent viral proteins are likely expressed during primary EBV infection, early EBV-specific CD8+ T cell responses during acute infection are directed towards lytic proteins (42-44). After resolution of the acute infection, lytic epitope-specific CD8+ T cells decline in frequency but most remain detectable throughout latency. Latent epitope-specific CD8+ T cells, however, are uncommonly detected in the peripheral blood at presentation with AIM (43) but become detectable after several weeks. Frequencies of latent epitope-
specific CD8\(^+\) T cells are generally lower than frequencies of lytic epitope-specific CD8\(^+\) T cells during AIM and remain stable over long periods of time. A graphical representation of the pattern of EBV lytic and latent epitope recognition by CD8\(^+\) T cell over the course of infection is shown in Figure 1.4.

EBV-specific CD8\(^+\) T cells play an important role in maintaining EBV latency. Under circumstances of T cell immunosuppression, EBV-induced B cell lymphoproliferative disorders can ensue. Treatment of EBV-induced lymphoproliferative disorders with infusions of \textit{in vitro} generated EBV-specific CD8\(^+\) T cell lines can elicit remission of lymphoma (45, 46).

3. \textit{EBV-specific CD4\(^+\) T cells}

While EBV-specific CD8\(^+\) T cell responses have been well-documented, EBV-specific CD4\(^+\) T cell responses have been less well studied, especially during AIM. Several groups have derived EBV-specific CD4\(^+\) T cell lines from latently-infected individuals and have used these lines to determine the EBV protein and epitope specificity of CD4\(^+\) T cell responses (47-50). For example, EBNA-1- and EBNA-3C-specific CD4\(^+\) T cell lines have been generated from healthy, EBV seropositive individuals (47, 48). In addition, Leen and colleagues have identified HLA class II-restricted EBNA-1, EBNA-3C, LMP-1, and LMP-2 epitopes using ELISPOT assays (51). EBNA-1-specific CD4\(^+\) T cell clones have been shown to inhibit outgrowth of EBV-infected B cells \textit{in vitro} (52). This is of interest, as all EBV-associated tumors express EBNA-1.
Figure 1.3

**EBV Lytic Peptide Epitopes**

<table>
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<tr>
<th>Peptide</th>
<th>Acute</th>
<th>Latent</th>
</tr>
</thead>
<tbody>
<tr>
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<td>23</td>
<td>27</td>
</tr>
<tr>
<td>A2 BZLF-1</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>A2gp350</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>A2gp85LV</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>A2gp85SV</td>
<td>8</td>
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<td>8</td>
<td>6</td>
</tr>
<tr>
<td>B7 BZLF-1</td>
<td>8</td>
<td>10*</td>
</tr>
<tr>
<td>A3 BRLF-1</td>
<td>8</td>
<td>10*</td>
</tr>
</tbody>
</table>

**EBV Latent Peptide Epitopes**

<table>
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<th>Peptide</th>
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</thead>
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</tr>
<tr>
<td>A2EBNA-3A</td>
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<tr>
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<td>12*</td>
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<tr>
<td>B7EBNA-3C</td>
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<td>8</td>
</tr>
<tr>
<td>A3EBNA-3A</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

% of Individuals Responding

Figure 1.4: EBV antigen-specific recognition by CD8+ T cells during acute and latent infection (from Catalina M.D. 2001 *J Immunol* 167:4450-4457). Previously defined, HLA class I-restricted EBV epitopes were used to stimulate whole blood of individuals with acute or persistent EBV infection, and intracellular IFN-γ was measured. Percentages of individuals with detectable frequencies of IFN-γ-producing cells are shown. Numbers represent n values.
Whereas most studies have defined CD4+ T cell responses specific for latent antigens during persistent EBV infection, little is known about the timing, magnitude, breadth, or specificity of CD4+ T cell responses during acute EBV infection. White et al. have reported the isolation of a BHRF-1-specific CD4+ CTL clone from the peripheral blood of an AIM patient (50), but more direct characterization of EBV-specific CD4+ T cell responses and their relationship to CD8+ T cell responses is limited.

E. Thesis Aims

The objective of this thesis dissertation was to examine EBV-specific CD4+ T cell responses over the course of acute infection. In an effort to gain insight into their in vivo effector functions, experiments were designed to characterize EBV-specific CD4+ T cells ex vivo, rather than using previously-employed in vitro culture approaches.

In Chapter 3, short in vitro stimulation assays followed by intracellular cytokine staining are used to characterize the timing, magnitude, and antigen-specificity of CD4+ T cells over the course of primary EBV infection. We hypothesize that, similar to EBV-specific CD8+ T cell responses during AIM, EBV-specific CD4+ T cell responses will be directed predominantly toward lytic proteins. However, we find that both lytic and latent protein-specific CD4+ T cells are readily detected at presentation with AIM but decline rapidly thereafter. Responses to BZLF-1, BMLF-1, and EBNA-3A are more commonly detected
than responses to EBNA-1. Responses decline rapidly following presentation with AIM and are undetectable by 6 mo to 1 yr thereafter.

The relationship between EBV-specific CD4+ T cells and both EBV-specific CD8+ T cells and blood viral load over the course of AIM are examined in Chapter 4. We hypothesize, based on observed differences between EBV lytic- and latent-specific CD4+ and CD8+ T cell responses early in AIM, that CD4+ and CD8+ T cell responses directed against the same lytic protein may also differ. Peptide-based assays are used for concurrent analysis of the timing, magnitude, and specificity of BZLF-1-specific CD4+ and CD8+ T cell responses. Our data show that BZLF-1-specific CD4+ T cell responses tend to peak earlier and at lower frequencies than BZLF-1-specific CD8+ T cell responses. EBV-specific CD4+ T cell responses correlate with blood viral load at presentation with AIM, and both decline in parallel over time.

Because of the rapid decline in EBV-specific CD4+ T cell responses following AIM, we further investigate their phenotypic and functional capabilities in Chapter 5. In order to test the hypothesis that intracellular IFN-γ secretion assays may underestimate EBV-specific CD4+ T cell frequencies, direct ex vivo staining with HLA class II tetramers is used. We report the detection of high frequencies of BZLF-1 epitope-specific CD4+ T cells at presentation with AIM but a significant reduction in the frequencies by 6 wk following presentation, despite persistently high peripheral blood viral loads. The frequencies of tetramer-staining cells exceed the frequencies of IFN-γ-secreting cells, and tetramer-
staining cells proliferate poorly in vitro. We propose that our data suggest a relationship between high viral burden and impaired virus-specific CD4\(^+\) T cell responses.
CHAPTER II
MATERIALS AND METHODS

A. Patient Population.

Individuals presenting to the clinic at the University of Massachusetts at Amherst Student Health Service (Amherst, MA) with clinical symptoms consistent with acute infectious mononucleosis and a positive monospot test were recruited for study. Following informed consent, 50ml of blood was collected at several time points, both at or shortly following presentation with symptoms, and at 6 mo to 1 yr thereafter. Acute EBV infection was confirmed by the detection of IgM antibodies to the EBV viral capsid antigen in patient sera.

Healthy EBV-seropositive and -seronegative donors were also recruited for study. EBV serological status was determined by the presence or absence of IgG antibodies to the viral capsid antigen in serum. Following informed consent, blood (~10ml) was collected periodically from these donors. All studies were approved by the Human Subjects Committee at the University of Massachusetts Medical School (Worcester, MA).

B. HLA typing.

HLA class II typing was determined by PCR. DNA was isolated from approximately 5 X 10^6 cells or 200μl whole blood using the QIAamp DNA Mini kit (Qiagen, Valencia, CA). For low resolution HLA-DR typing, the DRB SSP kit was
used (Biotest Diagnostics, Denville, NJ). For high resolution typing, the Olerup DRB1*01 SSP kit was used (Genovision, West Chester, PA).

C. Synthesis of GST-fusion proteins.

The plasmid encoding GST-tagged BZLF-1 was generously provided by Shannon Kenney (University of North Carolina, Chapel Hill, NC). Genes encoding BMLF-1 (aa 1-438), EBNA-1 (aa 363-641), and EBNA-3A (aa 403-812) were PCR amplified from B95.8 using the following primer sets: 5'-CGCGGGATCCCCCATGGAGGGCAGCGAAGAA-3' and 5'-CGCGCAATTGTTATTGATTTAATCCAGGAAC-3' for BMLF-1; 5'-CGCGGGATCCCCGGGGGAAGTCGTGAAGA-3' and 5'-CGCGGAATTCTTACTCCTGCCCTTCCTCACCC-3' for EBNA-1; and 5'-CGCGGGATCCCCGTAGAACCCTGCCTGTC-3' and 5'-CGCGGAATTCTTAGGCTCATCTGGAGGATC-3' for EBNA-3A. The amplified genes were cut by appropriate restriction enzymes and ligated into the multiple cloning site of the GST expression vector, pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ) to create an N-terminal GST fusion. Plasmids were used to transform *E. coli* strain BL21DE3RP (Stratagene, La Jolla, CA). This strain was also transformed with the pGEX-3X plasmid containing no insert for purification of GST. Transformed clones were grown to midlog phase at 37°C and induced with 0.6mM isopropyl-β-thiogalactosidase for 2-3h. Bacteria were resuspended in buffer containing protease inhibitor (50mM Tris HCL, 25%
sucrose, 1mM EDTA, 0.2mM phenylmethylsulfonyl fluoride, pH 8) and lysed by
the sequential addition of 1mg/ml lysozyme, 2mg/ml DNase I, 1M MgCl₂, and
lysis buffer (50mM Tris-HCL (pH 8), 1% Triton X-100, 100mM NaCl, 1% Sodium
dehoxycholate). Lysates were snap frozen in liquid N₂, clarified after thawing by
centrifugation at 17,400 x g, and incubated with glutathione sepharose 4B
(Amersham Pharmacia Biotech, Piscataway, NJ) overnight at 4°C.

GST and GST-fusion proteins were purified according to manufacturer’s
instructions using a batchwise method with small modifications. Suspensions
were centrifuged to sediment the matrix, and the supernatants were removed.
The glutathione sepharose 4B was washed with cold PBS until OD₂₈₀ of the wash
buffer was < 0.010. Protein was eluted with elution buffer (50mM Tris-HCL,
10mM reduced glutathione, pH 8) and PMSF was added at a final concentration
of 0.1mM. Protein from pooled eluates was quantified using Bradford Assay and
analyzed by SDS-PAGE and Western blot. Protein preparations were further
purified over polymyxin B columns (Pierce, Rockford, IL) to remove endotoxin
according to manufacturer’s instructions. Concentrated stocks were stored at
-20°C. Working stocks for immunology assays were diluted to 1mg/ml in RPMI
and stored at -20°C.

D. Peptides.

Sixteen 25-mer, overlapping (10aa) peptides spanning the BZLF-1 protein
and additional truncation peptides were synthesized for epitope mapping
(Peptide Core Facility, UMMS). Peptides were dissolved in DMSO and stored at -80°C. Working stocks were further diluted in RPMI to a final concentration of 1μg/μl and stored at 4°C. Peptide sequences are listed in Table 4.1. As all patients were HIV-negative, the A2-restricted Gag epitope (SLYNTVATL) was used as a negative control. The HLA-DR1-restricted peptide epitope QHYREVAAAKSSE (Genemed Synthesis, Inc., South San Francisco, CA) was dissolved in water and used for either HLA class II tetramer production or diluted to 1μg/μl for stimulation of cells.

E. Antigen and peptide stimulation of cells.

Heparinized, fresh whole blood or cryopreserved PBMC (0.5ml) was incubated with 20μg/ml GST or GST-fusion protein or 10μg/ml peptide at 37°C in 5% CO₂ for a total of 6 h. Except where noted, 3μg/ml each of anti-CD28 + anti-CD49d (BD PharMingen, San Diego, CA) was added for costimulation. As a positive control, staphylococcal enterotoxin B (SEB) (Toxin Technology, Sarasota, FL) at 2μg/ml was used. GolgiPlug (BD PharMingen San Diego, CA) was added for the final 4 h of incubation. Comparable responder cell frequencies were obtained using either fresh whole blood or cryopreserved cells as starting material.

F. Intracellular cytokine staining.
Intracellular staining for cytokine production was performed as previously described (53) with minor modifications. Following stimulation, EDTA was added at 2mM and the cells were vortexed repeatedly for 15 min. Red cells were lysed using FACS Lysing Solution (BD Biosciences, San Diego, CA) and washed in PBS containing 1% fetal calf serum (FCS). Cells were then permeabilized in FACSPerm (BD Biosciences, San Diego, CA), washed, and stained with a cocktail of antibodies appropriate for the experiment, including: CD8-FITC (Sigma-Aldrich, St. Louis, MO), CD4-PerCP, CD4-APC, CD3-PerCP, CD69-PE (all BD Biosciences), CD4-PE, CD45RO-FITC, CD45RA-FITC, IFN-γ-APC, TNF-α-APC, IL-2-APC, IL-4-PE, IL-5-PE, mip-1α-PE, and mip-1β-PE (all BD PharMingen).

Cytokine-secreting cells were analyzed by four-color flow cytometry on a BD Biosciences FACSort with added laser and CellQuest software (BD Biosciences, San Jose, CA). Isotype control antibodies and appropriate positive and negative controls were used to define cytokine-secreting populations. Approximately 50,000-100,000 events were collected through the lymphocyte gate as determined by forward vs. side scatter pattern. For cytokine secretion analysis in response to GST-fusion proteins, IFN-γ was plotted vs. CD69 using a CD4+CD45RO+ gate. No cytokine production was detected by CD4+CD45RO- cells in our assays. Frequencies of IFN-γ-secreting cells that were 2 SD above the mean background response to GST alone were considered significant (>0.06%). Cytokine production was not detected in response to EBV proteins in
EBV-seronegative individuals. Frequencies of IFN-γ-secreting cells in response to overlapping BZLF-1 peptides were determined by plotting IFN-γ vs. CD69 after gating on CD4+ or CD8+ populations. Frequencies of cytokine secreting cells that were 2 SD above the mean background response to the A2-Gag peptide were considered significant (>0.03% for CD4+ T cells; >0.04% for CD8+ T cells). For cytokine secretion analysis in response to the QHY peptide, cytokine was plotted vs. CD4 using a CD3+ gate. Frequencies of cytokine secreting cells were determined by subtracting the background response to the A2-Gag peptide.

G. Cytokine staining of tonsil cells.

Tonsils destined to be discarded from individuals undergoing routine tonsillectomy were obtained as approved by the Human Subjects Committee at the University of Massachusetts Medical School (Worcester, MA). Cells were teased from the tonsil tissue using a scalpel, filtered through a 70μm nylon cell strainer (BD Falcon, Franklin Lakes, NJ), and washed 3X in RPMI 1640 media supplemented with 10% FCS, 2mM L-glutamine, and 0.01mg/ml gentamicin (R10 media). Cells were cryopreserved until use.

Freshly thawed tonsil cells were stimulated with GST-fusion proteins as above, except the total incubation time was increased to 18 h, with the addition of GolgiPlug for the final 16 h. Intracellular IFN-γ production was detected and analyzed as above.
H. Real-time PCR quantification of EBV viral load.

EBV DNA copy frequency was estimated using a modification of the previously described real-time TaqMan PCR method for EBV quantification (54) or using LightCycler PCR. B lymphocytes were enriched either from whole blood using the RosetteSep protocol (StemCell Technologies Inc., Vancouver, BC, Canada) or from freshly thawed PBMC by MACS using a B cell isolation kit (Miltenyi Biotec, Auburn, CA). Genomic DNA was extracted using the DNAeasy kit (Qiagen, Valencia, CA).

The number of EBV copies was determined using the LightCycler-EBV Quantification Kit (Roche Diagnostics Corporation, Indianapolis, IN) or Taqman method. For the Taqman PCR method, the oligonucleotide primer-pairs amplify conserved sequences of the BALF5 region that encodes the viral DNA polymerase within the EBV genome, and their sequences are as follows: forward primer 5'-CGGAAGCCCTCTGGACTTC-3'; reverse primer 5'-CCCTGTTTATCCGATGGAATG-3'. A fluorogenic probe (5'-TGTACACGCACGAGAAATGCGCC-3'), which will hybridize to a sequence located within the region amplified by the forward and reverse primers, was synthesized with a FAM reporter molecule attached to the 5' end and a TAMRA quencher linked at the 3' end. The PCR mixture contained either 100ng or 200ng of genomic DNA in a 50ul reaction volume containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl, 5 mM MgCl2; 10mM EDTA; 100 uM each of dATP, dCTP, dGTP, and dTTP; 0.2mM of each primers; 0.1mM fluorogenic probe; and 1.25U of AmpliTaq
Gold DNA polymerase (PE Applied Biosystems, Foster City, CA). The amplification was performed in ABI TaqMan 7700 Sequence Detector thermocycler (PE Applied Biosystems) according to the following cycling parameters: 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase followed by 45 cycles of 15 sec at 95°C and 1 min at 62°C. DNA extracted from B cells from EBV-seropositive and -seronegative donors were included as positive and negative controls, respectively.

Serially diluted control plasmid (from $10^5$ to 10 copies) containing a single copy of the BALF5 amplicon generated by the forward and reverse primers was included in each PCR assay. The threshold cycle (CT) value corresponding to the point at which the real-time fluorescence exceeded 10 times the SD of the baseline for each sample was calculated for each sample. The CT values were then plotted against copy number to construct the standard curve, and from the CT values of test samples the EBV copy number was calculated using the software for data analysis (Sequence Detector version 1.6, PE Applied Biosystems). Samples with CT values that exceeded 45 cycles in our conditions of the assay were considered negative for EBV.

To determine the cellular equivalents of template DNA within each test sample, real-time PCR was performed on aliquots of template DNA to amplify a region within the duplicate-copy cellular chemokine receptor gene, CCR5. The primers used were 5'- GCTGTGGTTGCGTCTCTCCAGGA-3' (forward) and 5'-CTCACAGGCCCTGTGCTCTTCTTC-3' (reverse). The corresponding
fluorogenic oligonucleotide probe used for the Taqman method was 5'-FAM-AGCAGCGCAGGACCAGCCCCAAG-TAMRA 3'. Alternatively, LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics Corporation, Indianapolis, IN) was used to quantify CCR5. Serially diluted plasmid containing a single copy of the CCR5 gene was used to generate the standard curve and the CCR5 copy number in the sample was determined. The number of cellular equivalents was then calculated and the EBV copy frequency in the population of B-lymphocytes in each test sample was expressed as copies of EBV genome per $10^6$ B cells. Each sample was tested in triplicate, and the mean of the three values was considered for quantifying the EBV load.

I. Generation and maintenance of EBV-specific CD4$^+$ T cell lines.

Peptide-specific cell lines were generated using one of two methods. The first method involved peptide-specific stimulation and expansion. Cells were incubated at high cell concentration in a small volume with 100μg/ml peptide at 37°C in 5% CO₂ for 1h. The cells were then washed and plated in R10 media. After 2 d incubation, 50U/ml rhIL-2 (Roche Diagnostics Corporation, Indianapolis, IN) was added. Cells were maintained and expanded as necessary in R10 media supplemented with rhIL-2. Peptide specificity was confirmed by intracellular IFN-γ staining in response to appropriate peptide. Cell lines generated in this manner generally ceased dividing 2-4 wk following initial stimulation.
Alternatively, non-specific stimulation followed by isolation and expansion of peptide-specific cells was also used to generate peptide-specific CD4+ T cell lines. Cells were plated at 2 X 10^6/ml in R10 media supplemented with 50U/ml rhIL-2 and 1μg/ml of the bispecific monoclonal antibody anti-CD3:CD8 (provided by Johnson Wong, Massachusetts General Hospital, Boston, MA). This antibody leads to an expansion of CD3+CD4+ T cells with a selective loss of CD8+ T cells (55). After a sufficient number of CD4+ T cells were expanded, the cells were stimulated with peptide, and IFN-γ-secreting cells were isolated by MACS using the IFN-γ secretion assay-cell enrichment and detection kit (Miltenyi Biotec, Auburn, CA). Isolated, peptide-specific cells were plated in R10 media supplemented with 50U/ml rhIL-2 and irradiated, nonautologous PBMC (feeder cells). Cells were maintained and expanded, as necessary, in rhIL-2-supplemented R10 media and peptide-specificity of the cell line was confirmed using intracellular IFN-γ staining in response to peptide stimulation. Subsequently, cells were restimulated every 2-3 wk in rhIL-2-supplemented R10 media containing feeder cells (cell line: feeder=1:3) and anti-CD3 (OKT-3, American Type Culture Collection, Manassas, VA). Cell lines generated in this manner could be maintained up to several months. Aliquots of cells were cryopreserved periodically for future use.

J. HLA restriction analysis.
In order to determine the HLA restriction of peptide-specific responses, HLA-matched and -mismatched BLCL were pulsed with peptide and used to stimulate peptide-specific CD4+ T cell lines for analysis of intracellular IFN-γ production. BLCL used were: LG2 (generously provided by Larry Stern, UMMS), which is homozygous for DRB1*0101 and E1135 (derived from an AIM patient), with genotype HLA-DRB1*0402, *1302; -DQB1*0302, *06041. BLCL were incubated with 10μM peptide for 1 h at 37°C in 5% CO₂. Peptide-pulsed BLCL were then washed 3X in R10 and combined with CD4+ T cells at a ratio of 10:1 (T cell line: BLCL) in a total volume of 1ml R10 media supplemented with 1μg/ml each of anti-CD28 + anti-CD49d. Some tubes were also incubated with anti-HLA-DR blocking antibody (BD PharMingen, San Diego, CA) or isotype control (Research Diagnostics Inc., Flanders, NJ) at 5μg/ml to differentiate between HLA-DR and -DP- or -DQ-restricted responses. Cells were incubated at 37°C in 5% CO₂ for a total of 7 h. GolgiPlug was added for the final 6 h of incubation. Following incubation, cells were placed at 4°C overnight. Intracellular IFN-γ staining was performed the following day as above.

K. HLA class II tetramers.

Biotinylated monomers of HLA-DRA1*0101/DRB1*0101 bound to either QHY peptide or Flu-HA peptide were generously provided by Larry Stern (UMMS). The monomer was tetramerized for use as a staining reagent by the stepwise addition of streptavidin(SA)-PE (Biosource, Camarillo, CA). The
optimal SA-PE: DR1 monomer ratio was determined as previously described (56) by titration followed by staining of the QHY-specific CD4+ T cell line. As a negative control, either DR1/Flu-HA tetramerized in an identical manner as DR1/QHY tetramer or PE-conjugated DR1/Clip tetramer was used (Beckman Coulter, Fullerton, CA).

Whole blood and cell lines were stained as described (57) with a few modifications. Cells were stained with 10μl tetramer in a total volume of 200μl in 5-ml polystyrene round bottom FACS tubes at 37°C in 5% CO₂ for 2h. Following tetramer staining, cells were placed on ice and stained for 20 min with CD3-PerCP, CD4-APC (both BD Biosciences), and CD8-FITC (Sigma-Aldrich), CD45RA-FITC, CD45RO-FITC, CD11a-FITC, CD25-FITC, CD27-FITC, CD28-FITC, CD62L-FITC, CD95-FITC, or CD154-FITC (all BD PharMingen). Appropriate isotype control antibodies were also included for gating purposes. For some experiments, cells were stained with CD4-PerCP (BD Biosciences) and a combination of FITC- and APC-conjugated CD45RA, CD45RO, CD11a, CD62L, and CD95 antibodies (BD PharMingen).

After antibody staining, cells were lysed with FACS Lysing Solution and washed twice with cold PBS containing 1% FCS. Cells were then fixed with 1% paraformaldehyde and analyzed by four-color flow cytometry. Approximately 150,000 events were collected through a live lymphocyte gate as determined by forward vs. side scatter pattern. For tetramer-binding frequency analysis, CD4 was plotted vs. tetramer on CD3+ gated cells. Quadrants were set using the
negative control DR1/Clip tetramer. For phenotypic analysis of tetramer-binding cells, phenotypic marker was plotted vs. tetramer on CD3⁺CD4⁺ gated cells. Quadrants were set using DR1/Clip tetramer, isotype control antibodies, and careful assessment of positively- and negatively-staining non-tetramer-binding populations.

L. Cellular proliferation measured by CFSE

Cryopreserved PBMC were thawed rapidly and washed in R10 media and then washed twice in PBS. CFSE (Sigma, St. Louis, MO) was added at a final concentration of 0.01μM at room temperature for 5 min to label all cells. FCS was added at 20% (v/v) to stop the reaction and the cells were washed twice in 2% FCS/PBS. Cells were then plated at 1-2 X 10⁶/ml in R10 media supplemented with 10μg/ml peptide as indicated, with or without 10U/ml rhIL-2 (Roche Diagnostics Corporation, Indianapolis, IN) in 24-well plates. As a positive control, anti-CD3 (OKT-3, American Type Culture Collection, Manassas, VA) and IL-2 were added. Approximately 5 X 10⁵ cells were harvested at several timepoints thereafter and stained with DR1/QHY tetramer as above to measure proliferation of QHY-specific CD4⁺ T cells indicated by reduced CFSE fluorescence.
CHAPTER III

EBV-SPECIFIC CD4$^+$ T CELLS TARGET BOTH LYTIC AND LATENT EBV PROTEINS EARLY IN ACUTE INFECTIOUS MONONUCLEOSIS

A. Introduction.

Prior to these studies, little was known about the timing, magnitude, breadth, or specificity of CD4$^+$ T cell responses during acute EBV infection. Most of the previous reports described EBV-specific CD4$^+$ T cell responses using long-term in vitro culture methods. Studies generally focused on responses targeting EBV latent proteins in individuals with established infection. However, much more was known about EBV-specific CD8$^+$ T cell responses over the course of acute EBV infection. Namely, lytic epitope-specific CD8$^+$ T cells were detected more commonly and at generally higher frequency than latent epitope-specific CD8$^+$ T cells early in AIM. Over time, lytic epitope-specific CD8$^+$ T cells declined in frequency and latent epitope-specific CD8$^+$ T cells were more frequently detected.

Based on this differential pattern of antigen recognition by CD8$^+$ T cells during acute EBV infection, we hypothesized that there may be differential antigen expression or processing and presentation of lytic and latent EBV proteins. In order to better characterize EBV-specific CD4$^+$ T cells during AIM and test whether there is a similar pattern of lytic and latent antigen recognition
by CD4+ T cells over the course of AIM, EBV lytic and latent protein antigens were produced for use in stimulation assays to detect antigen-specific IFN-γ production by CD4+ T cells in the peripheral blood of individuals with AIM.

B. *EBV lytic and latent protein antigens.*

Since our lab (43) and others (42, 44) have previously shown that EBV lytic (BZLF-1, BMLF-1) and latent (EBNA-3A) proteins are commonly recognized by CD8+ T cells, and recognition of EBNA-1 by CD4+ T cells had been recently reported (48, 51), we prioritized these proteins for study. Truncations of the genes encoding these proteins were cloned into a GST expression vector, produced in *E. coli*, and purified. A Western blot of the GST-fusion proteins is shown in Figure 3.1. In addition to the band visualized at the expected molecular weight for each full-length fusion protein, bands of intermediate size are also present, indicating the production of incomplete fusion proteins. As the proteins were ultimately destined for uptake, processing, and presentation by antigen presenting cells in our stimulation assays, the presence of truncated proteins within the antigen preparations was acceptable. In the last step of purification, endotoxin was removed to reduce nonspecific stimulation. GST alone was purified in an identical manner for use as a negative control stimulus.
Figure 3.1: Western blot of purified GST-tagged EBV proteins. Proteins were run on SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted with anti-GST antibody. Molecular weight markers, in kDa, are shown to the left. Z=GST-BZLF-1 (~53kDa), M=GST-BMLF1_{aa1-438} (~74kDa), E1=GST-EBNA-1_{aa363-541} (~57kDa), E3A=GST-EBNA-3A_{aa403-812} (~71kDa), GST (~26kDa).
C. Detection of EBV-specific CD4⁺ T cell responses early during AIM.

The frequencies of EBV-specific CD4⁺ T cells were measured in the peripheral blood of individuals at and following presentation with AIM using intracellular IFN-γ staining following in vitro stimulation with each soluble protein (Figure 3.2a). SEB was used as the positive control in these studies. Cytokine production was not detected following the in vitro stimulation of whole blood from healthy, EBV seronegative adults (Figure 3.2b). At presentation with AIM, EBV-specific CD4⁺ T cells were detected in all individuals (Table 3.1). Eight (89%) of 9 individuals had detectable responses to two or more proteins. Responses to BZLF-1 were detected most frequently (17 of 17 individuals studied). BMLF-1- and EBNA-3A-specific CD4⁺ T cell responses were each detected in the peripheral blood of 7 (78%) of 9 individuals studied. By contrast, EBNA-1-specific CD4⁺ T cells were detected in only 3 (33%) of 9 individuals studied.

Frequencies of CD4⁺ T cells producing IFN-γ in response to each EBV protein are shown in Figure 3.3. At presentation, the frequencies of CD4⁺ T cell responses to BZLF-1 were generally higher than those directed at the other proteins. BZLF-1-specific CD4⁺ T cell responses were measured at frequencies that ranged from 0.08% to 1.75% of CD4⁺CD45RO⁺ cells (median = 0.31%). BMLF-1-specific CD4⁺ T cell responses were measured at frequencies ranging from 0.07% to 0.54% (median = 0.27%), while EBNA-3A-specific CD4⁺ T cell responses ranged from 0.08% to 0.34% (median = 0.16%). EBNA-1-specific CD4⁺ T cell responses ranged from 0.1%-0.36% (median = 0.23%).
Figure 3.2

A.

E-1126 (AIM)

BZLF-1

SEB

EBNA-1

EBNA-3A

GST

B.

E-024 (EBV Negative)

BZLF-1

SEB

EBNA-1

EBNA-3A

GST
Figure 3.2: Intracellular IFNγ staining of EBV-specific CD4⁺ T cells. Whole blood from a patient presenting with AIM (a) or a healthy EBV seronegative adult (b) was stimulated with GST-tagged fusion proteins BZLF-1, BMLF-1, EBNA-1, or EBNA-3A or GST alone as a negative control. Stimulation with SEB served as a positive control. Antigen-specific cells are expressed as a percent of CD4⁺CD45RO⁺ cells co-expressing CD69⁺IFN-γ⁺.
Table 3.1

<table>
<thead>
<tr>
<th>Visit</th>
<th>BZLF-1</th>
<th>BMLF-1</th>
<th>EBNA-1</th>
<th>EBNA-3A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%(17/17)</td>
<td>78%(7/9)</td>
<td>33%(3/9)</td>
<td>78%(7/9)</td>
</tr>
<tr>
<td>3wk</td>
<td>72%(13/18)</td>
<td>56%(5/9)</td>
<td>33%(3/9)</td>
<td>56%(5/9)</td>
</tr>
<tr>
<td>6wk</td>
<td>61%(11/18)</td>
<td>33%(3/9)</td>
<td>22%(2/9)</td>
<td>33%(3/9)</td>
</tr>
<tr>
<td>6mo</td>
<td>25%(4/16)</td>
<td>0%(0/8)</td>
<td>0%(0/8)</td>
<td>0%(0/8)</td>
</tr>
<tr>
<td>1yr</td>
<td>0%(0/11)</td>
<td>0%(0/5)</td>
<td>0%(0/4)</td>
<td>0%(0/5)</td>
</tr>
</tbody>
</table>

Table 3.1: Percentage of individuals with detectable EBV-specific CD4+ T cell responses over the course of AIM. In parenthesis, the numerator indicates the number of individuals with detectable CD4+ T cell responses; the denominator indicates the total number of individuals tested at each visit.
Figure 3.3

% CD4⁺ CD45RO⁺ Cells

BZLF-1

EBNA-1

BMLF-1

EBNA-3A

(1.75%)
**Figure 3.3:** EBV-specific CD4⁺ T cell frequencies following presentation with AIM. Frequencies of IFN-γ-producing cells in response to BZLF-1 (upper left), BMLF-1 (lower left), EBNA-1 (upper right), and EBNA-3A (lower right) were calculated as in Fig. 3.2 for each individual and plotted versus study timepoint. Each character represents an individual patient. Solid lines indicate median responder cell frequency of detectable responses. The hatched line in each plot represents the limit of detection (0.06% of CD4⁺CD45RO⁺ cells).
D. EBV-specific CD4⁺ T cell responses decline rapidly following acute infection.

Blood was collected at sequential timepoints up to 1 yr following presentation with AIM to determine the stability of EBV-specific CD4⁺ T cell responses over time following acute EBV infection. As shown in Table 3.1 and Figure 3.3, the numbers of individuals responding and the responder cell frequencies of BZLF-1-, BMLF-1-, EBNA-1-, and EBNA-3A-specific CD4⁺ T cells were highest within 3 wk of presentation with AIM and declined rapidly over time following primary infection. By 6 wk following presentation, BZLF-1 responses were detected in only 61% of individuals, BMLF-1 and EBNA-3A responses in only 33% of individuals, and EBNA-1 responses in only 22% of individuals. Median responder cell frequencies in individuals with detectable responses dropped to 0.12%, 0.07%, 0.09%, and 0.09% for BZLF-1, BMLF-1, EBNA-1, and EBNA-3A, respectively by the 6 wk timepoint. EBV-specific CD4⁺ T cells were undetectable in most individuals by 6 mo and in all individuals by 1 yr following AIM. Of interest, EBV-specific responses were not detected in any of twelve EBV seropositive healthy adults tested (data not shown).

To determine whether other cytokines were produced in response to these EBV antigens over the course of infection, we assayed for IL-4 production by intracellular cytokine staining and by ELISPOT assay. IL-4 production was not detected by intracellular cytokine staining or ELISPOT assay in response to in vitro stimulation with BZLF-1, BMLF-1, EBNA-1, or EBNA-3A proteins at any time point from presentation through convalescence (data not shown).
E. Rare detection of EBV-specific CD4\(^+\) T cell responses from tonsil cells.

Because EBV-specific CD4\(^+\) T cells were readily detected in the peripheral blood during AIM but not detected after resolution of acute infection, we hypothesized that the cells could be trafficking to tissues. As the tonsils are the site of initial infection and periodic reactivation of the virus and are therefore a logical homing site for virus-specific cells, we obtained tonsils from individuals undergoing routine tonsillectomy to determine whether we could detect EBV-specific CD4\(^+\) T cell responses. EBV infection of tonsil cells was determined by real-time Taqman PCR. Nine of 13 tonsil samples (69\%) were EBV positive by PCR (Table 3.2).

Stimulation of isolated tonsil cells with the EBV fusion proteins for a total of 6 hr did not lead to significant frequencies of CD4\(^+\) cells producing IFN-\(\gamma\); however, a few samples showed a low frequency of IFN-\(\gamma\)-producing CD4\(^+\) T cells that fell just below our level of detection. Therefore, the total incubation time was increased to 18 hr to allow longer stimulation and potentially increase detection of EBV-specific CD4\(^+\) T cells. After incubation for the increased time period, we were able to detect significant IFN-\(\gamma\) production from only 1 of 9 (11\%) EBV positive tonsil samples. This response was specific for EBNA-1 (Figure 3.4). As negative controls, IFN-\(\gamma\) production was not detected from EBV negative samples or in response to GST alone (Table 3.2 and Figure 3.4).
Table 3.2

<table>
<thead>
<tr>
<th>EBV Status</th>
<th>Sample</th>
<th>EBV DNA Copies/10^6 Cells</th>
<th>EBV-specific CD4^+ T Cell Response?</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV +</td>
<td>T-010</td>
<td>6.15E+02</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T-011</td>
<td>5.11E+04</td>
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<td>T-012</td>
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<td>No</td>
</tr>
<tr>
<td></td>
<td>T-015</td>
<td>5.03E+02</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T-016</td>
<td>2.84E+03</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T-017</td>
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<td>T-021</td>
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<td>No</td>
</tr>
<tr>
<td></td>
<td>T-023</td>
<td>3.27E+05</td>
<td>No</td>
</tr>
<tr>
<td>EBV -</td>
<td>T-014</td>
<td>0.00E+00</td>
<td>No</td>
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<td>T-022</td>
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<tr>
<td></td>
<td>T-025</td>
<td>0.00E+00</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 3.2: EBV-specific CD4^+ T cell responses from isolated tonsil cells. Intracellular IFN-γ was measured as in Fig. 3.4 and responses >0.06% were considered positive. EBV load was measured by Taqman PCR.
Figure 3.4

A. T-010

B. T-022
Figure 3.4: Intracellular IFNγ staining of EBV-specific CD4⁺ T cells from tonsils. Cells isolated from an EBV PCR+ (a) or EBV PCR- (b) tonsil were stimulated with GST-tagged fusion proteins BZLF-1, BMLF-1, EBNA-1, or EBNA-3A or GST alone as a negative control for 18 h. Antigen-specific cells are expressed as a percent of CD4⁺ cells co-expressing CD69⁺IFN-γ⁺.
F. Discussion

In this chapter, the magnitude, specificity, and kinetics of circulating EBV-specific CD4\(^+\) T cells were measured from the peripheral blood of individuals over the course of AIM. We hypothesized that differential recognition of lytic and latent EBV proteins by CD8\(^+\) T cells reflected differences in the presentation of these proteins to T cells and therefore we would detect a similar pattern of recognition by CD4\(^+\) T cells over the course of AIM. CD4\(^+\) T cells were stimulated with both lytic and latent EBV protein antigens to detect intracellular IFN-\(\gamma\) production. In contrast with previous studies demonstrating differential recognition of lytic and latent antigens by EBV-specific CD8\(^+\) T cells early in AIM, CD4\(^+\) T cell responses specific to both lytic and latent EBV proteins were detected at presentation with AIM. These results suggest that lytic and latent antigens are expressed very early during infection but are differentially recognized by CD4\(^+\) and CD8\(^+\) T cells. Responses to BZLF-1, BMLF-1, and EBNA-3A were more commonly detected than responses to EBNA-1. Responses were highest at presentation with AIM but declined rapidly thereafter and were undetectable by 6 mo to 1 yr after presentation.

Because we could not detect EBV-specific CD4\(^+\) T cells in the peripheral blood during viral latency, we hypothesized that they were homing to the tonsil. However, we were unable to detect EBV-specific CD4\(^+\) T cells in the majority of tonsil samples from individuals with EBV infection. Although these results do not support this hypothesis, it is possible that another method, such as direct
staining, may be required for detection of EBV-specific CD4$^+$ T cells in tonsil cells.
CHAPTER IV

CD4+ AND CD8+ T CELL RESPONSES AGAINST AN IMMEDIATE EARLY EBV PROTEIN DIFFER IN MAGNITUDE, KINETICS, SPECIFICITY, AND RELATIONSHIP WITH VIRAL LOAD OVER THE COURSE OF AIM

A. Introduction

In the previous chapter, we found that CD4+ T cell responses, unlike CD8+ T cell responses, were directed against EBV lytic and latent proteins. This suggests that the priming of EBV antigen-specific CD4+ and CD8+ T cells may differ. We therefore hypothesized that differences would be observed between CD4+ and CD8+ T cell responses directed against the same EBV protein. Additionally, we proposed that we would detect differences in the relationship between EBV-specific CD4+ and CD8+ T cell responses and viral load.

B. Detection of EBV-specific CD4+ and CD8+ T cell responses during AIM

In order to examine potential relationships between the magnitude, kinetics, and specificity of EBV-specific CD4+ and CD8+ T cell responses over the course of AIM, intracellular cytokine staining was performed after stimulation with overlapping peptides spanning the immediate early protein, BZLF-1 (peptide sequences are listed in Table 4.1). This lytic cycle protein was chosen because of our previous results showing that CD4+ T cells commonly target BZLF-1 during
<table>
<thead>
<tr>
<th>Peptide number</th>
<th>Sequence</th>
<th>E-1121</th>
<th>E-1122</th>
<th>E-1125</th>
<th>E-1126</th>
<th>E-1127</th>
<th>E-1131</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MMDP NSTSEDVKFTPDPYQVPFVQA</td>
<td>8*</td>
<td>8*</td>
<td>8</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DPLYQVFVQAFDQATRVYQDGLGGS</td>
<td>8*</td>
<td>4, 8*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RVYQDLGGPSQAPLPCVLWPVLP EP</td>
<td>4*</td>
<td>8*</td>
<td>4*, 8</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CVLWPVLPEPLPQGQLTAYHVSTAP</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>LTAYHVSTAPTGWSFSAPQPA PENA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>SAPQPA PENAYQAYAAPQLFPVSDI</td>
<td>4</td>
<td>4*, 8</td>
<td>4, 8</td>
<td>8</td>
<td>8</td>
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</tr>
<tr>
<td>7</td>
<td>APQLFPVSDITQNQQTNQAGGEAPQ</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>TNQAGGEAPEQPQDGDNSTVQTA AAVF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>TVQTA AAVVFACPGANQGQLADIG</td>
<td>8</td>
<td></td>
<td>4</td>
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<td></td>
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</tr>
<tr>
<td>10</td>
<td>NQGQQLADIGVQPAPVAAPARR TR</td>
<td>4*</td>
<td></td>
<td></td>
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<tr>
<td>11</td>
<td>PVAAPARRTRKPKQQPESLEECDS E</td>
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<td>12</td>
<td>ESLEECDESELEIKRYKNRVAS RKR</td>
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<td>LLQHYREVAAAK SSENDRLRLLL KQ</td>
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<td>15</td>
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<tr>
<td>16</td>
<td>MCPS LDVDSIIPRTDPVLHEDLLNF</td>
<td>8</td>
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<td>8</td>
</tr>
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</table>
Table 4.1: Overlapping BZLF-1 peptides. Intracellular IFN-γ production by CD4⁺ cells and CD8⁺ cells in response to each peptide was measured in 6 patients with AIM over the first 6 wk of infection. A detectable CD4⁺ T cell response is denoted with a 4; a detectable CD8⁺ T cell response is denoted with an 8. An asterisk indicates a response to both the indicated peptide and the next consecutive peptide, suggesting the epitope may reside in the shared amino acid overlap.
AIM. Overlapping peptides were used as stimulation because the fusion protein did not stimulate CD8$^+$ T cells very well. Frequencies of peptide-specific cells were determined by IFN-γ production in response to each peptide after gating on CD4$^+$ or CD8$^+$ cells. As depicted in Tables 4.1 and 4.2, BZLF-1-specific CD4$^+$ and CD8$^+$ T cell responses were detected in all six individuals studied.

C. Magnitude of BZLF-1-specific CD4$^+$ and CD8$^+$ T cell responses during AIM. In order to quantify the response to the entire BZLF-1 protein, responses to individual, nonconsecutive peptides were added together. The sum of the peak frequencies of BZLF-1 peptide-specific CD8$^+$ T cells were substantially greater than the sum of the peak frequencies of BZLF-1 peptide-specific CD4$^+$ T cells in 4 of 6 individuals (Table 4.2) over the first 6 wk of AIM. BZLF-1-specific CD4$^+$ T cell responses measured by this method ranged from 0.06% to 0.71% (median = 0.24%) of CD4$^+$ cells. BZLF-1-specific CD8$^+$ T cell responses ranged from 0.06% to 3.55% (median = 1.97%) of CD8$^+$ cells.

D. Differential kinetics of BZLF-1-specific CD4$^+$ and CD8$^+$ T cell responses over the course of AIM. Consistent with our previous findings, BZLF-1-specific CD4$^+$ T cells were readily detected at presentation with AIM and declined rapidly thereafter (Table 4.2 and Figure 4.1). By contrast, BZLF-1-specific CD8$^+$ T cells, though also detected at presentation, increased in frequency following presentation with AIM,
Table 4.2: BZLF-1-specific CD4$^+$ and CD8$^+$ T cell responses following stimulation with overlapping peptides. For each patient, the number of nonconsecutive peptides eliciting a significant IFN-γ response was determined at presentation and throughout the first 6 wk. Frequencies shown represent the sum of the highest frequencies of IFN-γ-producing cells to all nonconsecutive peptides at presentation and throughout the first 6 wk.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4$^+$ T Cells</th>
<th>CD8$^+$ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Presentation</td>
<td>Total 6wk</td>
</tr>
<tr>
<td></td>
<td>#Peptides</td>
<td>Frequency</td>
</tr>
<tr>
<td>E-1121</td>
<td>4</td>
<td>0.37%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-1122</td>
<td>5</td>
<td>0.71%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-1125</td>
<td>1</td>
<td>0.11%</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-1126</td>
<td>5</td>
<td>0.43%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-1127</td>
<td>1</td>
<td>0.06%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-1131</td>
<td>1</td>
<td>0.11%</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
such that peak peptide-specific CD8\(^+\) T cell frequencies occurred 3 to 6 wk after presentation.

E. Specificity of BZLF-1-specific CD4\(^+\) and CD8\(^+\) T cell responses over the course of AIM.

In addition to the observed differences in the magnitude and kinetics of BZLF-1-specific CD4\(^+\) and CD8\(^+\) T cell responses, the regions of the BZLF-1 protein targeted by CD4\(^+\) and CD8\(^+\) T cells within an individual were different (Table 4.1 and Figure 4.1). In most individuals, multiple BZLF-1 peptides were targeted by both CD4\(^+\) and CD8\(^+\) T cells (Tables 4.1 and 4.2). Consistent with previous studies (42, 58), overlapping peptide 13 containing the HLA-B8-restricted RAK epitope was recognized by HLA-B8\(^+\) patients, E-1126 and E-1127 during AIM.

F. EBV-specific CD4\(^+\) T cell responses correlate with blood viral load during AIM.

In order to examine the relationship between EBV load and EBV-specific CD4\(^+\) T cell responses, viral load was quantitated over the course of AIM using real-time PCR (Figure 4.2). We found that EBV load was highest at presentation with AIM (range: 1.13 \times 10^4 to 2.28 \times 10^6 copies/10^6 B cells; median: 1.47 \times 10^5 copies/10^6 B cells, n=9). To determine whether EBV load at presentation correlated with antigen-specific CD4\(^+\) T cell frequency, log viral load was plotted against the frequency of CD4\(^+\) T cells specific for BZLF-1, BMLF-1, EBNA-1, and
Figure 4.1

E-1121

E-1126

%Specific CD4'T Cells

%Specific CD8'T Cells

BZLF-1 Peptide
Figure 4.1: Frequency and specificity of BZLF-1-specific CD4$^+$ and CD8$^+$ T cells following acute EBV infection. Intracellular IFN-γ staining was performed after stimulation with each of the 16 overlapping peptides of BZLF-1 (peptide sequences are listed in Table 4.1). Frequencies of CD69$^+$IFN-γ$^+$ cells were calculated after gating on either CD4$^+$ or CD8$^+$ T cell populations. The solid horizontal line in each plot represents the level of detection of the assay using peptides (0.03% for CD4$^+$ cells, 0.04% for CD8$^+$ cells). Note the different y-axis scale for CD4$^+$ versus CD8$^+$ cells. Representative data from 2 individuals are shown.
Figure 4.2

% CD4+CD45RO+ Cells

EBV Copies/10^6 B Cells

Time (weeks)
Figure 4.2: EBV viral load and EBV-specific CD4+ T cell responses in 9 individuals over the course of AIM. Viral load was determined by Taqman PCR of DNA extracted from B cell enriched populations. Antigen-specific CD4+ T cell frequency was determined as in Figure 3.2. The solid line in each plot represents the level of detection of the assay (0.06% of CD4+CD45RO+ cells).
EBNA-3A (Figure 4.3). While BMLF-1-EBNA-1-, and EBNA-3A-specific CD4+ T cell frequency correlated with EBV load at presentation (R=0.56, 0.45, and 0.59, respectively), a weaker correlation was observed between viral load and BZLF-1-specific CD4+ T cell frequency (R=0.34). However, it should be noted that one patient (E-1122) appeared to be an outlier; upon exclusion of this patient, reanalysis of the data revealed a good correlation between viral load and EBV-specific CD4+ T cell frequency at presentation with AIM (R=0.63, 0.55, 0.54, and 0.59 for BZLF-1, BMLF-1, EBNA-1, and EBNA-3A, respectively).

Viral load dropped rapidly following presentation with AIM, with the median viral load dropping 2 log_{10} by 3 wk following presentation. EBV-specific CD4+ T cell responses dropped in parallel with viral load, suggesting that these responses were antigen-dependent. While the frequencies of BZLF-1-specific CD8+ T cells also correlated with blood viral load at presentation with AIM (Figure 4.4), BZLF-1-specific CD8+ T cell frequencies subsequently increased in most individuals as viral load declined.

G. Discussion

Results presented in this chapter show differences in the magnitude, kinetics, and specificity of CD4+ and CD8+ T cell responses against the lytic protein, BZLF-1. This result supports our hypothesis that differences would be observed between CD4+ and CD8+ T cell responses directed against the same
Figure 4.3: Relationship between the frequencies of EBV-specific CD4\(^+\) T cell responses and viral load at presentation with AIM. Antigen-specific CD4\(^+\) T cell frequency was determined as in Figure 3.2. Viral load was determined by Taqman PCR of DNA extracted from B cell enriched populations. R values represent correlation coefficients.
Figure 4.4: Relationship between the frequencies of BZLF-1-specific CD8⁺ T cell responses and viral load at presentation with AIM. Frequencies represent the sum of the highest frequencies of IFN-γ-producing cells to all nonconsecutive peptides at presentation. Viral load was determined by Taqman PCR of DNA extracted from B cell enriched populations. R values represent correlation coefficients.
protein. However, these differences could reflect intrinsic differences between CD4\(^+\) and CD8\(^+\) T cells and/or differences in their priming.

BZLF-1-specific CD4\(^+\) T cell responses tended to peak earlier and at lower frequencies than BZLF-1-specific CD8\(^+\) T cell responses, and were not persistently detectable in most individuals 6 wk or more following presentation with AIM. By contrast, BZLF-1-specific CD8\(^+\) T cell responses remained high through 6 wk following presentation. Within an individual, CD4\(^+\) and CD8\(^+\) T cell responses targeted different regions of the BZLF-1 protein. However, it is important to note that BZLF-1 was recognized by both CD4\(^+\) and CD8\(^+\) T cells in all six individuals studied, suggesting that this protein is very immunogenic.

Additionally, the relationship between viral load and T cell responses was examined. BZLF-1-, BMLF-1-, EBNA-1-, and EBNA-3A-specific CD4\(^+\) T cell responses as well as BZLF-1-specific CD8\(^+\) T cell responses correlated with blood viral load at presentation with AIM. However, while BZLF-1-specific CD8\(^+\) T cell responses remained high or increased following presentation, both EBV-specific CD4\(^+\) T cell responses and viral load decreased immediately thereafter. This may indicate that CD4\(^+\) T cell responses are more dependent on the presence of viral antigen.
CHAPTER V

DIRECT ENUMERATION AND CHARACTERIZATION OF EBV EPITOPE-SPECIFIC CD4+ T CELLS

A. Introduction

In the previous chapter, we detected differences between BZLF-1-specific CD4+ and CD8+ T cell responses and found that EBV-specific CD4+ T cell responses correlated with blood viral load. In Chapters III and IV, we found that EBV-specific CD4+ T cell responses declined rapidly and, unlike EBV-specific CD8+ T cell responses, were undetectable by intracellular IFN-γ staining by 3 wk to 6 mo following presentation with AIM. However, we hypothesized that intracellular IFN-γ secretion assays may underestimate EBV-specific CD4+ T cell frequencies. Therefore, HLA class II tetramers are used in this chapter to examine the frequency and functionality EBV-specific CD4+ T cells over the course of AIM.

B. Identification of BZLF-1 epitope-specific CD4+ T cell responses.

From our studies using overlapping peptides spanning the BZLF-1 protein to detect intracellular IFN-γ production by CD4+ T cells from patients during AIM, we identified several BZLF-1 peptide-specific CD4+ T cell responses. In order to characterize these responses in greater detail, CD4+ T cell lines specific for two
peptides were generated from cryopreserved PBMC obtained at presentation with AIM. A BZLF-1 peptide #14-specific CD4+ T cell line was generated from patient E-1125, and a BZLF-1 peptide #15-specific CD4+ T cell line was generated from patient E-1126. Both peptide-specific CD4+ T cell lines retained specificity for their respective peptides (Figure 5.1). HLA DR typing of both patients is listed in Table 5.1.

In order to better define the epitopes recognized within the 25-mer peptides, truncated peptides were synthesized. Stimulation of the CD4+ T cell lines with the truncated peptides followed by intracellular IFN-γ staining was used to fine map the epitopes. By this method, it was determined that the peptide #14-specific CD4+ T cell line generated from patient E-1125 recognized the peptide QHYREVAAAKSSE (aa 198-210) as well as BZLF-1 peptide #14 (data not shown). We also mapped the peptide #15-specific CD4+ T cell line generated from patient E-1126 to NDRLRLLLKQMC (aa 211-222) (data not shown). These two peptide-specific CD4+ T cell responses will be referred to as QHY-specific and NDR-specific responses.

C. The QHY-specific CD4+ T cell response is restricted by HLA-DR1.

Upon close examination of the QHY peptide sequence, it was noted that it contained a predicted binding motif for HLA-DR1. As patient E-1125 is HLA-DR1+ (HLA-DRB1*0101), we wanted to test whether this response was, in fact, restricted by HLA-DR1. Therefore, we performed intracellular IFN-γ assays using
Figure 5.1

A.

E1125  T cell line

0.10%  73%

CD69  IFN-γ

B.

E1126  T cell line

0.12%  93%

CD69  IFN-γ

BZLF #14

Negative control

BZLF #15

Negative control
**Figure 5.1:** BZLF-1 peptide-specific CD4⁺ T cell lines derived from AIM patients. Intracellular IFN-γ staining of whole blood at presentation with AIM or derived T cell line following stimulation with BZLF-1 peptide #14 for patient E-1125 (a) or BZLF-1 peptide #15 for patient E-1126 (b) or A2-restricted HIV Gag peptide as a negative control. Peptide-specific cells are expressed as a percent of CD4⁺ cells co-expressing CD69⁺IFN-γ⁺.
Table 5.1

<table>
<thead>
<tr>
<th>Patient</th>
<th>HLA-DRB1</th>
<th>Frequency of CD3⁺CD4⁺ QHY tetramer⁺ cells at presentation with AIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-1125</td>
<td>*0101, 0407</td>
<td>0.2%↑</td>
</tr>
<tr>
<td>E-1126</td>
<td>*0701, 1303</td>
<td>ND</td>
</tr>
<tr>
<td>E-1182</td>
<td>*11XX/13XX, 11XX/14XX</td>
<td>0%</td>
</tr>
<tr>
<td>E-1183</td>
<td>*0101, 13XX</td>
<td>1.7%</td>
</tr>
<tr>
<td>E-1184</td>
<td>*04XX, 12XX</td>
<td>0%</td>
</tr>
<tr>
<td>E-1185</td>
<td>*0101, 03XX</td>
<td>1.9%</td>
</tr>
<tr>
<td>E-1187</td>
<td>*0103, 08XX</td>
<td>0.02%</td>
</tr>
<tr>
<td>E-1188</td>
<td>*0101, 04XX</td>
<td>1.1%</td>
</tr>
</tbody>
</table>

**Table 5.1:** HLA-DR typing and QHY tetramer staining of selected AIM patients. Tetramer staining was performed on fresh, whole blood. ↑ indicates that tetramer staining of patient E-1125 was performed on cryopreserved PBMC. ND= not done.
peptide-pulsed, HLA class II-matched and -mismatched BLCL as antigen presenting cells. We also used a HLA-DR blocking antibody to distinguish between HLA-DR- and -DP- or -DQ-restricted responses. Based on these experiments, it was determined that the QHY-specific CD4\(^+\) T cell line was indeed restricted by HLA-DR1 (Figure 5.2).

D. QHY-specific CD4\(^+\) T cells can be directly stained with an HLA-DR1 tetramer.

In order to directly stain QHY-specific CD4\(^+\) T cells, we tetramerized an HLA-DR1 monomer loaded with the QHY peptide that was generously provided by Dr. Larry Stern. Staining of the QHY-specific CD4\(^+\) T cell line with this tetramer revealed that 63% of the CD4\(^+\) T cells in this line stained with tetramer (Figure 5.3). This frequency was comparable to the frequency of CD4\(^+\) T cells that produced IFN-\(\gamma\) in response to the QHY peptide (Figure 5.1). As a negative control, the QHY-specific CD4\(^+\) T cell line did not stain with an HLA-DR1 tetramer loaded with the Flu HA\(_{306-318}\) peptide (Figure 5.3). As an additional control, the NDR-specific CD4\(^+\) T cell line did not stain with the QHY tetramer.

E. QHY-specific CD4\(^+\) T cells are detected at high frequency during AIM.

To determine whether QHY-specific CD4\(^+\) T cells were generated by other HLA DR1\(^+\) individuals during AIM, blood was collected at presentation and at 1, 2, 3, 4, and 6 wk thereafter. HLA-DR1\(^+\) individuals were identified using low-resolution HLA typing by DNA PCR using sequence-specific primers. Fresh,
Figure 5.2: HLA restriction analysis of the QHY-specific response. Intracellular IFN-γ staining was performed after incubation of the QHY-specific CD4+ T cell line with QHY peptide-pulsed, HLA class II-matched or -mismatched BLCL. An HLA-DR-blocking antibody or isotype control was used to confirm HLA-DR restriction. Frequencies of CD4+ cells co-expressing CD69+IFN-γ+ are shown.
Figure 5.3: Tetramer staining of the QHY-specific CD4⁺ T cell line. Frequencies of tetramer-binding cells are expressed as a percent of CD3⁺CD4⁺ cells. Staining of the QHY-specific CD4⁺ T cell line with Flu HA tetramer and of the NDR-specific CD4⁺ T cell line with QHY tetramer were included as specificity controls.
whole blood was stained with QHY tetramer at each timepoint (Figure 5.4). At presentation, QHY-specific CD4⁺ T cells were detected at high frequencies (about 1-2% of CD4⁺ T cells) in three HLA-DR1⁺ individuals studied. QHY-specific CD4⁺ T cells declined over time, such that by 6 wk following presentation they were only detected in 2 of 3 individuals at frequencies between 0.3-0.5% of CD4⁺ T cells. Controls were included to confirm specificity of tetramer staining (Figure 5.4). CD4⁺ T cells from an HLA-DR1⁺ individual did not stain with an HLA-DR1 tetramer loaded with the self-peptide, CLIP. Additionally, CD4⁺ T cells from three HLA-DR1⁻ individuals with AIM did not stain with the QHY tetramer.

F. QHY-specific CD4⁺ T cells decline over time following AIM despite persistently high viral load.

Because of the rapid decline in QHY-specific CD4⁺ T cells following presentation with AIM, we serially quantified blood EBV load to determine whether the decline in QHY-specific CD4⁺ T cells could be attributed to a similar reduction in viral burden. Surprisingly, we found that EBV load remained high in all three individuals studied (Figure 5.5) over 6 wk following presentation with AIM.
Figure 5.4: QHY-specific CD4⁺ T cells over the course of AIM. Tetramer staining was performed on fresh, whole blood. Representative QHY tetramer staining of an HLA-DR1⁺ AIM patient (E-1183) at presentation is shown. Staining of patient E-1183 with Clip tetramer and of an HLA-DR1⁻ patient (E-1184) with QHY tetramer were included as specificity controls. The lower right panel shows the mean frequency of CD3⁺CD4⁺-gated QHY-tetramer⁺ cells in 3 patients over the course of AIM. Error bars represent SD of replicate tetramer stains.
Figure 5.5

%CD3+CD4+ Cells

EBV Copies/10^6 B Cells

Time (days)
Figure 5.5: Tetramer staining, IFN-γ production, and viral load over the course of AIM. QHY-tetramer staining (○) and intracellular IFN-γ staining in response to QHY peptide stimulation (△) were performed on fresh, whole blood. Viral load (□) was determined by LightCycler PCR of DNA extracted from B cell enriched populations.
G. Frequencies of QHY-specific CD4+ T cells detected by tetramer staining exceed frequencies detected by IFN-γ production.

The frequencies of EBV-specific CD4+ T cells detected by tetramer staining exceeded the frequencies that we had previously detected using flow cytometry-based IFN-γ secretion assays. Thus, we went on to measure and compare the frequencies of tetramer-staining cells with IFN-γ-secreting cells (Figure 5.5). We performed tetramer and intracellular IFN-γ staining in parallel using fresh, whole blood. The frequencies of IFN-γ-producing CD4+ T cells detected were similar to those originally measured ex vivo and used to define the QHY epitope. In addition, they are comparable to the frequency reported for CD4+ T cells specific for an epitope derived from the lytic protein, GP350 (59). However, the frequency of IFN-γ production in response to QHY peptide was markedly lower than the frequencies of tetramer-staining cells.

We had previously detected EBV-specific IFN-γ production only from the CD45RO+ subset of CD4+ cells (60), but recently IFN-γ production by GP350-specific CD4+ T cells expressing CD45RA has been shown (59). We therefore co-stained with CD45RA in our intracellular IFN-γ assays and found that nearly all QHY-specific, IFN-γ-producing CD4+ T cells were CD45RA negative (data not shown). This was noteworthy because a high percent of QHY tetramer+ CD4+ T cells co-expressed CD45RA (50-70% of tetramer+ cells, see below).
H. Intracellular staining for alternative cytokine production

In order to determine whether QHY-specific CD4+ T cells produce cytokines other than IFN-γ in responses to stimulation with QHY peptide, we stimulated cryopreserved PBMC and co-stained for IL-2, IL-4, IL-5, mip-1α, and mip-1β. CD4+ T cells did not reproducibly produce any of these cytokines in response to stimulation with QHY peptide.

I. Low proliferative potential of QHY-specific CD4+ T cells in vitro at presentation with AIM

Because we detected a high frequency of QHY tetramer+ cells that did not appear to secrete IFN-γ, we wanted to further examine their ability to proliferate using peptide-induced proliferation and CFSE labeling. In preliminary experiments using cryopreserved PBMC samples from presentation with AIM, we found that QHY-specific CD4+ T cells did not proliferate well in response to peptide (Figure 5.6). In fact, the frequency of QHY tetramer+ cells declined after incubation with QHY peptide for 5 d. Addition of 10U/ml IL-2 to the culture did not restore proliferative capacity (data not shown). However, cells from 6 wk post-presentation appeared to proliferate in response to QHY peptide slightly better. Interpretation of this data is limited, though, because of low cell number due to sample availability. Further studies using fresh PBMC are required to demonstrate a reproducible proliferative defect.
**Figure 5.6:** *In vitro* proliferative capacity of QHY-specific CD4⁺ T cells over the course of AIM. Cryopreserved PBMC from patient E-1183 at presentation (visit 1) or 6 wk thereafter (visit 6) were labeled with CFSE and incubated with QHY peptide for 5 d. CFSE intensity is shown after gating on Tetramer⁺CD3⁺CD4⁺ cells for days 1 (light gray) and 5 (dark gray) of culture with peptide.
J. Distribution of phenotypic markers on QHY-specific CD4+ T cells is heterogeneous and similar to that found on general CD4+ T cell population

In our tetramer staining experiments, we co-stained with a number of phenotypic markers to gain insight into phenotype, homing properties, and sensitivity to cell death. These markers included CD45RA and RO isoforms, CD28, CD27, CD25, CD40L, L-selectin, LFA-1, and Fas. We found that the frequency of QHY-specific CD4+ T cells co-expressing most of these markers resembled the frequency of all CD4+ T cells that expressed the given marker. Specifically, most CD4+ T cells (including QHY-specific CD4+ T cells) during AIM were heterogeneous for CD45 isoforms, CD28+, CD27+, CD25-, CD40L-, L-selectinhi, LFA-1lo, and up to half expressed Fas. A potential difference in CD45RA expression between QHY-specific CD4+ T cells and the CD4+ T cell population in general was noted. A higher frequency of QHY-specific CD4+ T cells co-expressed CD45RA. This difference tended to be greatest at the earliest timepoints during AIM.

K. QHY-specific CD4+ T cells decline over the year following AIM.

In order to determine whether QHY-specific CD4+ T cells were maintained long-term after AIM, we tetramer stained cryopreserved PBMC from HLA-DR1+ individuals at timepoints up to 1 yr following presentation with AIM (Figure 5.7). Tetramer staining of cryopreserved PBMC showed lower frequencies of QHY-specific CD4+ T cells than had been previously found using fresh, whole blood at
Figure 5.7: QHY-specific CD4$^+$ T cells through 1 yr following AIM. Cryopreserved PBMC from HLA-DR1$^+$ individuals (n=4) up to 1 yr were thawed and stained with QHY-tetramer. Frequencies of tetramer-binding cells are expressed as a percent of CD3$^+$CD4$^+$ cells. Time 0 represents presentation with AIM.
presentation (~0.2% of CD3+CD4+ cells). This was not surprising, as we found loss of tetramer staining due to cryopreservation in some fresh vs. frozen comparison experiments (data not shown). By 3 mo to 1 yr following AIM, frequencies of QHY-specific CD4+ T cells were generally very low to undetectable.

L. Discussion

In this chapter, we hypothesized that EBV-specific CD4+ T cells could be more accurately quantified by direct staining with HLA class II tetramers. A CD4+ T cell epitope (QHY) derived from the EBV lytic protein, BZLF-1, was identified and shown to be restricted by HLA-DR1. HLA-DR1 tetrameric reagents loaded with the QHY peptide specifically stained both QHY-specific CD4+ T cell lines and QHY-specific CD4+ T cells ex vivo from individuals with AIM. Frequencies of QHY-specific CD4+ T cells were very high at presentation with AIM, but declined rapidly over time despite persistently high viral load. The kinetics of this response is similar to the kinetics of lytic epitope-specific CD8+ T cells detected by tetramer over the first several weeks following AIM (43). QHY-specific CD4+ T cells were undetectable in 1 of 3 HLA-DR1+ individuals by 6 wk and 2 of 2 HLA-DR1+ individuals by 1 yr following presentation with AIM.

It is likely that functional assays, such as IFN-γ production, underestimate the fréquences of epitope-specific CD4+ T cells. Early during acute infection, when there is a high viral burden, QHY-specific CD4+ T cells appear to be
"stunned" or anergic as determined by lack of IFN-γ production or low proliferative potential. While it is possible that in vitro manipulation of highly in vivo-stimulated T cells may compromise their functional activity (61, 62), this may also reflect a potential mechanism by which the virus is able to establish lifelong persistence.
CHAPTER VI

DISCUSSION

A. Thesis overview

Prior to the current study, little was known about CD4+ T cell responses during acute EBV infection. As CD4+ T cells play an important role in virus-specific immunity, especially in the context of persistent infections, the specificity, timing, magnitude, and functional properties of EBV-specific CD4+ T cells over the course of primary EBV infection were investigated. Our hypothesis at initiation of this study was that EBV-specific CD4+ T cells would be detected during AIM, with responses targeting primarily lytic cycle proteins at the earliest time-points studied.

Data presented in Chapter 3 using intracellular cytokine staining experiments revealed that, as expected, EBV-specific CD4+ T cell responses were generated in all individuals (n=17) during AIM. However, these responses targeted viral proteins expressed during both the lytic and latent cycle of the virus life cycle. These data were unexpected and show that the antigen recognition pattern of CD4+ and CD8+ T cells is different during AIM. While EBV-specific CD4+ T cells were detected at measurable frequencies (up to 2.7% of CD4+CD45RO+ cells) early in infection, responses declined rapidly over the first few weeks following presentation with AIM.
In Chapter 4, relationships between EBV-specific CD4+ T cell responses and both EBV-specific CD8+ T cell responses and EBV load over the course of AIM were examined. We hypothesized that differences would be observed between CD4+ and CD8+ T cell responses directed against the same EBV protein. We found that both CD4+ and CD8+ T cell responses specific for BZLF-1 were detected; however, the magnitude of CD4+ T cell responses was lower than CD8+ T cell responses. Additionally, the kinetics of BZLF-1-specific CD4+ and CD8+ T cell responses differed. EBV-specific CD4+ T cell responses declined in parallel with EBV load over time following presentation with AIM, suggesting that these responses may be antigen-dependent.

Finally, in Chapter 5, BZLF-1 epitope-specific CD4+ T cells were detected directly ex vivo by HLA class II tetramers from HLA-DR1+ individuals with AIM. We hypothesized that intracellular IFN-γ secretion assays may underestimate EBV-specific CD4+ T cell frequencies. Indeed, we found that frequencies of tetramer-staining cells far exceeded frequencies detected by peptide-induced IFN-γ production. Moreover, tetramer-staining cells did not proliferate well in response to peptide stimulation in vitro. These apparent functional impairments may, in part, explain our inability to detect EBV-specific CD4+ T cells at later time-points by intracellular IFN-γ. However, frequencies of tetramer-staining cells also decrease over time and are not detected in a small number of HLA-DR1+ individuals by 6 mo to 1 yr following AIM. Data from this chapter suggest a
relationship between acute EBV infection and impaired EBV-specific CD4⁺ T cell responses.

B. EBV infection as a system for studying human virus-specific T cell responses

Primary EBV infection is an attractive system for studying the generation and maintenance of virus-specific T cells in humans because acutely infected individuals can be readily identified at presentation with symptoms of AIM. Using this system, the frequencies and specificities of EBV antigen-specific CD8⁺ T cell responses have been well characterized (42-44); however, less is known regarding EBV-specific CD4⁺ T cell responses over the course of infection. We therefore undertook the present study in order to characterize the kinetics, specificity, and maintenance of EBV-specific CD4⁺ T cell responses and to compare these responses with EBV-specific CD8⁺ T cell responses and blood viral load. Marked differences in the expansion, specificity, and stability of EBV-specific CD4⁺ and CD8⁺ T cells were observed over time.

The marked lymphocytosis observed during AIM involves an expansion of both CD4⁺ and CD8⁺ T cells (63). It has recently been shown that the expansion of CD8⁺ T cells is largely antigen-specific (42, 43). The recent discovery of an EBV-induced superantigen suggests that the CD4⁺ T cell expansion may, in part, be non-specifically driven (64). However, our data document that at least 2.7% of circulating effector/memory CD4⁺ T cells are EBV-specific. Moreover, this response is broadly specific in most individuals and is directed at both lytic and
latent EBV antigens. This study constitutes the first ex vivo demonstration of the antigen-specificity of CD4⁺ T cells during primary EBV infection.

The early detection of EBV-specific CD4⁺ T cells during acute EBV infection in this study are consistent with a previous study documenting the early detection of CMV-specific CD4⁺ T cells during primary CMV infection in kidney transplant recipients (40). Frequencies of CMV-specific CD4⁺ T cells peaked very early (median 7 d) after detection of CMV DNA and declined rapidly thereafter. However, CMV-specific CD4⁺ T cells are readily detected in the peripheral blood of convalescent individuals (40, 53) even though CMV DNA is not commonly detected in the peripheral blood of healthy CMV carriers (65). Differences in the biology of these persistent viruses, such as the location and degree of antigen expression or the availability of antigen for processing and presentation, may strongly influence the virus-specific memory T cell pool.

Our findings bear some similarities but important differences from the results of studies of acute viral infections in mice. Murine studies of LCMV infection have demonstrated three general phases of T cell responses [reviewed in (66)]. During the expansion phase, the clonal expansion of virus-specific CD8⁺ T cells appears to precede and exceed that of virus-specific CD4⁺ T cells. Following clearance of LCMV (a non-persistent virus), virus-specific CD8⁺ T cells undergo a more pronounced reduction in frequency than virus-specific CD4⁺ T cells (contraction phase). After the contraction phase, virus-specific CD8⁺ T cell frequencies appear to be stable, while virus-specific CD4⁺ T cell frequencies
decline (67). Our data are similar to the murine LCMV data in that they demonstrate a less robust expansion of virus-specific CD4+ T cells than CD8+ T cells, a phenomenon likely related to the slower rate of CD4+ T cell expansion documented in in vitro and in vivo studies (66). Of interest, however, is that EBV-specific CD4+ T cell responses appear to be less well maintained than those generated during non-persistent infection with LCMV.

C. Lytic vs. latent EBV gene recognition

CD4+ T cell recognition of both lytic and latent proteins during acute EBV infection in Chapter 3 contrasts with the preferential recognition of lytic proteins by CD8+ T cells (42-44). Latent protein-specific CD8+ T cells are infrequently detected at presentation with AIM but become detectable after a few weeks. Some have suggested that the differences in the pattern of responses to EBV proteins over time are due to differences in the timing of expression of these proteins. Our data demonstrating the detection of CD4+ T cells specific for both lytic and latent proteins at presentation with AIM suggest that the predominance of lytic-specific over latent-specific CD8+ T cells during acute EBV infection cannot be accounted for simply by differences in antigen expression. These differences more likely reflect differences in the availability of these proteins to the appropriate antigen presenting cells or processing pathways for CD4+ and CD8+ T cells during acute infection.
One model to explain the differences in antigen recognition by CD4\(^+\) and CD8\(^+\) T cells is that the antigen presenting cell type for presentation of lytic and latent EBV proteins is different for CD4\(^+\) and CD8\(^+\) T cells. For example, latent proteins would most likely be expressed in B cells, but not in other infected cell types. Because EBV drives latently infected B cells into germinal center reactions, CD4\(^+\) T cells surrounding these areas may have better access to latent protein-expressing B cells than CD8\(^+\) T cells that do not enter this area. On the other hand, lytic proteins would likely be taken up by dendritic cells and presented to both CD4\(^+\) and CD8\(^+\) T cells. Alternatively, the antigen presenting cell type may affect the priming of CD4\(^+\) and CD8\(^+\) T cells. For example, B cell priming of latent-specific CD8\(^+\) T cells may be different than lytic-specific CD8\(^+\) T cell priming by other antigen presenting cells, such as dendritic cells.

Another model for the lytic vs. latent antigen recognition differences is the amount of these antigens present and/or the ability of these antigens to be presented on HLA class I and class II molecules. There is likely a higher amount of lytic proteins than latent proteins present during an acute infection because lytic, but not latent, cycle proteins are highly expressed. Latent protein expression is also tightly regulated. Therefore, CD8\(^+\) T cell responses targeting lytic proteins may predominate simply because of their greater abundance on HLA class I molecules. Additionally, the processing of EBV lytic and latent proteins may differ in efficiency. An example of this possibility is the EBNA-1 protein, which contains a Gly-Ala repeat region that inhibits its proteasomal
processing and subsequent presentation on HLA class I molecules (68). However, this does not affect presentation of this protein on HLA class II molecules, demonstrating a divergence in antigen-specific priming of CD4+ and CD8+ T cells.

A final argument for the observed antigen recognition pattern is that our whole blood and PBMC assays detect late-stage, circulating EBV-specific T cells that are in the process of migrating to another site in the body. Therefore, differences in the magnitude and kinetics of lytic- and latent-specific CD4+ and CD8+ T cells could reflect different homing and re-circulation properties of these cells. In MHV-68 infection of mice, the kinetics of detection of both latent-specific CD8+ T cells and lytic-specific CD4+ and CD8+ T cells were very similar in three tissues studied: bronchoalveolar lavage, mediastinal lymph node, and spleen (69). However, the magnitude of these responses in each tissue was different. Interestingly, lytic-specific CD8+ T cell responses peaked earlier than latent-specific responses. This suggests that the kinetic differences between lytic- and latent-specific CD8+ T cell responses are similar in mice and humans despite the incubation period between EBV infection and presentation with symptoms in humans.

D. EBV-specific CD4+ and CD8+ T cell responses

By examining both CD4+ and CD8+ T cell IFN-γ responses against BZLF-1 during AIM, we demonstrated differences in the expansion and stability of BZLF-
1-specific CD4+ and CD8+ T cells. Measured frequencies of BZLF-1-specific CD4+ T cells were highest at presentation but the rate of detection and the frequencies of detectable BZLF-1-specific CD4+ T cells declined over the first 6 wk of study. EBV-specific CD4+ T cell frequencies generally paralleled peripheral blood viral load, a finding that is consistent with the greater dependence of CD4+ T cell proliferation on antigen exposure in vitro (70, 71). By contrast, BZLF-1-specific CD8+ T cell frequencies continued to increase in most individuals over the weeks following presentation and remain detectable during convalescence. While the frequencies of BZLF-1-specific CD8+ T cells correlated with blood viral load at presentation with AIM, BZLF-1-specific CD8+ T cell frequencies subsequently increased in most individuals as viral load declined, suggesting that the expansion and maintenance of the CD8+ T cell population does not require persistently high levels of antigen (72).

The difference in kinetics of IFN-γ responses between CD4+ and CD8+ T cells against the same protein suggests that either the priming of these cells is different or that CD4+ and CD8+ T cells are intrinsically different in the kinetics of their expansion or functional capabilities. Based on their general functions of regulation (CD4+) and elimination of virus (CD8+) during an immune response, it is logical that CD8+ T cells would expand to higher frequencies than CD4+ T cells. The difference in the kinetics of detection of BZLF-1-specific CD4+ and CD8+ T cells could reflect the migration of these cells out of infected tissue. Alternatively, BZLF-1-specific CD8+ T cells may be very highly activated at early
time-points and therefore be less effective at secreting IFN-γ in response to in vitro stimulation than at later time-points.

It is very interesting that so many peptides within BZLF-1 are recognized by CD4+ and CD8+ T cells. While the HLA restriction of each peptide-specific response was not determined, it is possible that some of the responses are cross-reactive. A potential consequence of the multi peptide-specific BZLF-1 responses is an inability of the virus to mutate to escape the host response. In fact, the EBV genome is very well conserved, with only two different strains. Moreover, sequence differences between these strains reside in only a few viral genes, predominantly in EBNA-3. By sequence conservation, EBV may actually benefit from host responses that eliminate pathogenic EBV outgrowth and drive EBV persistence in resting memory B cells.

By tetramer staining, we found that the kinetics of CD4+ T cells specific for the BZLF-1 QHY epitope during AIM are similar to the kinetics of lytic epitope-specific CD8+ T cells, which are very high in frequency early but are greatly culled following AIM (42, 43). Differential maintenance of lytic epitope-specific CD8+ T cells into long-term memory has been noted (43). Interestingly, QHY-specific CD4+ T cells are detected in 2 of 3 of our HLA-DR1+ AIM patients by tetramer staining 6 wk after presentation with AIM. Future studies will determine whether QHY-specific memory CD4+ T cells are generated and persist for life akin to previously defined lytic epitope-specific CD8+ T cells.
While memory CD8+ T cells specific for both lytic and latent epitopes persist throughout viral latency, we are unable to detect EBV-specific CD4+ T cells by IFN-γ secretion after 6 mo to 1 yr following acute infection. There are several potential reasons why we are unable to do so. The frequency of memory EBV-specific CD4+ T cells may be below the limits of detection of intracellular IFN-γ assays. This possibility is supported by several published studies reporting the detection of EBV-specific CD4+ T cells during EBV latency (47-49, 51, 73), many of which required the generation of EBV-specific CD4+ T cell lines from healthy EBV carriers by long-term in vitro culture. Recently, Paludan and colleagues reported detection of EBNA-1-specific CD4+ T cells in the peripheral blood of a small number of healthy EBV carriers by intracellular cytokine staining at frequencies ranging from 0.24% to 0.66% of CD4+ T cells (74). However, we have been unable to replicate these findings despite the fact that our EBNA-1 construct (aa 363-641), like their EBNA-1 construct (aa458-641), contains defined CD4+ T cell epitopes.

As we have shown in Chapter 5, high frequencies of EBV epitope-specific CD4+ T cells detected by direct staining with HLA class II tetramers have impaired IFN-γ and proliferative responses. While we do not yet have tetramers to detect cells specific for other lytic and latent epitopes, it is possible that EBV-specific memory CD4+ T cells persist but do not secrete IFN-γ, and thus are undetectable in our assays. Frequencies of circulating EBV-specific CD4+ T cells may also be below the limit of detection using HLA class II tetramers. Currently,
*in vitro* culture methods may allow for better detection and expansion of this cell population, but it is important to note that functional responses of CD4\(^+\) T cell lines do not necessarily reflect *ex vivo* or *in vivo* effector capabilities.

EBV-specific CD4\(^+\) T cells may be sequestered at sites of viral reactivation and replication such as the tonsil. Preliminary intracellular IFN-\(\gamma\) assays using cells from tonsillar tissue have not supported this hypothesis; however, additional functional studies and tetramer staining experiments are needed to further investigate this possibility.

E. Direct *ex vivo* staining of EBV-specific CD4\(^+\) T cells

While HLA class II tetramer staining of CD4\(^+\) T cell lines has been reported (56, 75), this is among the first studies to show direct *ex vivo* staining of human virus-specific CD4\(^+\) T cells over the course of an acute infection. High frequencies (0.01-3.1%, n=3) of *Borrelia*-specific CD4\(^+\) T cells from synovial fluid of treatment-resistant Lyme arthritis patients have been reported (76). More recently, HCV-specific CD4\(^+\) T cells were magnetically isolated from patients who resolved HCV viremia using HLA-DR4 tetramers (77). These cells were very low in frequency and represented memory HCV-specific CD4\(^+\) T cells. Tetramer staining of influenza-specific CD4\(^+\) T cells within 60 days of immunization has also been recently reported (78). Frequencies of influenza-specific CD4\(^+\) T cells in that study [ranging from 1 in 30,000 (0.003%) to 1 in 600 (0.17%) CD4\(^+\) T cells]
were lower than we report here, most likely because of differences between acute viral infection and vaccination.

Phenotypically, QHY-specific CD4\(^+\) T cells were heterogeneous and did not differ significantly from the general CD4\(^+\) T cell population. The exception was CD45RA, which was more frequently expressed on tetramer-staining cells. Recently, a phenotypic analysis of CD4\(^+\) T cells that produce IFN-\(\gamma\) in response to lytic (GP350) and latent (EBNA-3C) peptides during primary EBV infection was performed (59). We find, as was reported for GP350-specific CD4\(^+\) T cells, that QHY-specific CD4\(^+\) T cells are CD27\(^+\) and CD28\(^+\). However, GP350-specific CD4\(^+\) T cells, detected by IFN-\(\gamma\) staining, were mostly CD45RO\(^+\), with a minority CD45RA\(^+\) (59). We find that a majority of QHY-specific CD4\(^+\) T cells detected by tetramer staining express CD45RA, but QHY-specific, IFN-\(\gamma\)-producing CD4\(^+\) T cells are also nearly exclusively CD45RO\(^+\). These differences likely reflect marker expression differences between directly stained cells and the subset of cells that produce IFN-\(\gamma\) in response to peptide stimulation. Alternatively, the epitope specificity may influence CD45 isoform expression.

F. EBV-specific CD4\(^+\) T cell responses and EBV load

In Chapter 4, we found that EBV-specific CD4\(^+\) T cell responses and EBV load generally declined in most individuals over time following AIM. This suggested that CD4\(^+\) T cell responses were antigen-dependent. In Chapter 5,
QHY-tetramer\(^+\) CD4\(^+\) T cells in the three HLA-DR1\(^+\) AIM patients studied also declined in frequency over time despite a sustained, high EBV load.

It is important to note that EBV load, quantitated by DNA PCR, may not be an accurate reflection of viral gene expression. There is evidence to suggest that expression of EBV lytic proteins declines following AIM. Prang et al. detected BZLF-1 transcripts and protein in B cells from individuals with AIM; however, the percent of B cells expressing lytic proteins decreased after the first week of AIM (79). BZLF-1 transcripts, but not protein, were detected in healthy, EBV seropositive individuals. Therefore, the decline in EBV-specific CD4\(^+\) T cells may reflect a decrease in antigen expression following presentation with AIM.

G. Functional impairment of EBV-specific CD4\(^+\) T cells during acute infection?

The frequency of IFN-\(\gamma\) production in response to QHY peptide was markedly lower than the frequencies of QHY tetramer-staining cells. Low frequencies of tetramer positive CD8\(^+\) T cells that produce IFN-\(\gamma\) in response to peptide stimulation during AIM have been noted previously (61, 62). It is possible that EBV-specific T cells are so highly activated \textit{in vivo} that some are incapable of IFN-\(\gamma\) production upon restimulation \textit{in vitro}. However, whereas the percentage of tetramer-binding CD8\(^+\) T cells producing IFN-\(\gamma\) in response to peptide stimulation increased over time following AIM (62), the proportion of QHY-specific, IFN-\(\gamma\) producing CD4\(^+\) T cells remained consistently low over time.
A relationship between high viral load and low IFN-γ production by CMV-specific CD4+ T cells has also been reported. In acutely infected children with a sustained period of viral shedding in urine, lower frequencies of IFN-γ-producing CMV-specific CD4+ T cells were detected than from acutely infected adults with no viral shedding in urine (80). In primary CMV infection of transplant recipients, CMV-specific, IFN-γ-producing CD4+ T cells were detected much later after virus detection for symptomatic patients when compared with asymptomatic patients (38). While there was no statistically significant difference between maximum viral loads in symptomatic vs. asymptomatic patients, symptomatic patients tended to have higher viral loads. These data, along with ours, suggest that impaired IFN-γ responses are a characteristic of high viral burden during acute infection. It is unclear, however, whether low IFN-γ responses of CD4+ T cells result in, or are a consequence of high viral loads.

We also found that QHY-specific CD4+ T cells did not proliferate well in response to peptide. Impaired virus-specific CD4+ T cell proliferation during high viremia and chronic infection has been noted for several human viral infections. Chronic hepatitis B virus (HBV)-infected individuals have low HBV-specific CD4+ T cell proliferative responses, but regain proliferative capacity following treatment with lamivudine, a potent inhibitor of HBV replication (81). Studies have also documented an inverse correlation between HIV-1 viremia and HIV-specific CD4+ T cell proliferation (82, 83). Recently, proliferation of HIV epitope-specific CD4+ T cells from individuals with high HIV-1 viremia was found to be impaired, but could
be rescued by addition of exogenous IL-2 (84). Our demonstration that QHY-specific CD4\(^+\) T cells from individuals with high EBV loads also show impaired proliferation suggests that this relationship is common across many chronic viral infections.

There are several potential mechanisms by which EBV-specific CD4\(^+\) T cells may become unresponsive to stimulation. One possibility is that EBV-encoded immune modulators may interfere with the priming of EBV-specific CD4\(^+\) T cells. For example, the BCRF-1 protein is a homolog of human IL-10, which is an immunosuppressive cytokine. BCRF-1 has been shown to down-regulate expression of HLA molecules and co-stimulatory molecules on antigen presenting cells even in the presence of IFN-\(\gamma\) (85). Inefficient co-stimulation may lead to the production of EBV-specific CD4\(^+\) T cells that lack effector function.

Regulatory T cells are also induced in the presence of IL-10 (86). It is therefore possible that EBV-specific CD4\(^+\) T cells are inhibited by regulatory T cells. Recently, LMP-1-specific CD4\(^+\) T cells from EBV seropositive individuals were shown to secrete IL-10 and inhibit bystander proliferative and IFN-\(\gamma\) responses to mitogen and recall antigen stimulation (87). While BZLF-1-specific CD4\(^+\) T cells may be suppressed by regulatory T cells, we do not believe that they are regulatory T cells. This is based on our inability to detect IL-10 production in response to stimulation and due to their lack of CD25 expression, which is characteristic of regulatory T cells.
H. Conclusion

In conclusion, EBV lytic and latent antigen-specific CD4+ T cells are commonly detected during early EBV infection but differ in their kinetics and specificity from EBV-specific CD8+ T cell responses. High frequencies of EBV lytic epitope-specific CD4+ T cells were detected by HLA class II tetramer staining ex vivo over the course of AIM. Frequencies detected by tetramer staining exceeded those detected by production of IFN-γ in response to peptide, suggesting that functional assays can underestimate the true frequency of epitope-specific CD4+ T cells.

Early during acute infection, when there is a high viral burden, QHY-specific CD4+ T cells appear to be "stunned" or anergic as determined by lack of IFN-γ production or low proliferative potential. Whether this is due to the in vitro manipulation required for our functional assays or reflects a potential mechanism by which the virus is able to establish lifelong persistence requires further investigation.

A better understanding of how EBV-specific CD4+ T cells contribute to resolution of acute infection and/or the establishment of EBV latency has important implications for prevention and treatment of EBV infection. This thesis dissertation provides data identifying EBV protein and epitope targets of CD4+ T cells during acute infection. While, in some cases, the function of EBV epitope-specific CD4+ T cells may be altered or impaired during natural infection, vaccination and immunotherapeutic strategies aimed at generating strong
responses against these EBV proteins could prove to be effective at preventing EBV infection and/or treating EBV-associated disease. Additionally, identification of epitope targets allow for more detailed analyses of the role of CD4+ T cell responses during infection using reagents such as HLA class II tetramers. Differences between EBV-specific CD4+ and CD8+ T cell responses may provide insight into the priming of these cells during acute infection in vivo and suggest mechanisms by which EBV has evolved to manipulate host responses and avoid elimination.
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