# Prospective analysis of the primary immune response during naturally acquired Epstein-Barr virus infection in humans

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## Dedication

This thesis is dedication to God for everything he has done, for everything he is doing, and for everything he will do, for the people he has put in my life, both in the past, present and future.

**Abstract**

Apart from altering normal cellular function, cytopathic viruses cause cell lysis and injury while non-cytopathic viruses may lead to latent infections and subsequent complications. While both innate and adaptive immune responses are activated by infection, the population of virus-specific cytotoxic T-lymphocytes (CTL) is composed mostly of CD8 T cells. During a viral infection, naïve CD8 T cells undergo massive proliferation and differentiation. Several studies suggest that most of the activated proliferating cells are virus-specific, although there may be a role for activation of bystander CD8 T cells and cross-reactive CD8 T cells. Activated CD8 T cells undergo differentiation towards distinct functional pathways. Usually after elimination or sequestration of the pathogen, the majority of the activated cells will undergo apoptosis. The surviving fraction, estimated to be 5-20%, become antigen-specific memory T cells. Expansion of memory T cells into secondary effectors results in a more specific and faster recall, which is key during re-infection or control of persistent infections. In addition to CD8 T cells, other cells of the immune system, costimulatory signals and cytokines, play important roles in activating the immune system. Unfortunately, the activities of the immune system may injure tissue and result in immunopathological disease.

Infectious mononucleosis is one such example in which an exaggerated immune response to primary Epstein-Barr virus (EBV) infection is thought to cause disease. Little is known as to why EBV exposure commonly causes acute infectious mononucleosis (IM) in adults, but rarely in children, or why some adults (about 30%) asymptomatically seroconvert. One school of thought attributes disease to viral cytopathy while another suggests that development of disease is determined by the immune response to virus (i.e. CD8 lymphocytosis). The latter model suggests that lymphocytosis is a result of activated cross-reactive memory cells, which may increase in frequency with age. However, there is no evidence that pre-existing memory T cells become activated during

primary EBV infection. One limitation is that most of our understanding of acute EBV infection has relied on clinical studies of patients undergoing symptomatic disease.

Using primary EBV infections in humans as a model, we showed that a prospective study allows for capture of a broad range of symptomatic and asymptomatic individuals. Lymphocyte expansion was associated with disease severity during primary EBV infection and lymphocytosis was also associated with viremia. In reconciling the published data, we suggest that viral load in the blood drives NK and CD8 T cell expansion.

Moreover, we used several markers (CD38, HLA-DR, and granzyme B) to determine if pre-existing memory CD8 T cells became activated during acute infectious mononucleosis. We found that primary EBV infection resulted in bystander activation of pre-existing influenza-specific and cytomegalovirus-specific CD8 T cells. However, these cells did not generally expand and therefore, the CD8 lymphocytosis observed during acute EBV infection was composed largely of EBV-specific cells. Finally, in contrast to the mouse model of infectious mononucleosis, in humans, primary EBV infection did not result in attrition of bystander influenza and cytomegalovirus memory CD8 T cells.

These findings outline the significance of studying immunity in humans, because our results differed from what mouse studies would have predicted. These finding also illustrate the advantages of prospective studies for understanding pathogenesis including being able to observe a broader spectrum of disease expression. Finally, these results provide unique insight into the underlying immune response of primary EBV infection, and suggest potential strategies for controlling EBV-related disease such as targeting viral replication to control viral load and suppressing the immune response.

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# CHAPTER 1

## Introduction

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## EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) was discovered in 1964 by electron microscopy of suspension cultures of African Burkitt lymphoma cells [1]. Four years later, EBV was conclusively linked to infectious mononucleosis, which is its most common clinical manifestation [2]. The unifying and perplexing characteristic of human herpesviruses including EBV is that acquisition results in lifelong infection after the initial viral replication has been contained [3].

## THE BIOLOGY OF EBV

The biology of EBV including virus structure, genome, strain variability, replication and latency has been comprehensively reviewed elsewhere [4 5 6 7 8]. Briefly, we focus here on the areas that are crucial for understanding pathogenesis, diagnosis, treatment, and prevention of primary EBV infections. EBV, formally designated as human herpesvirus 4 (HHV-4), is one of the eight known human herpesviruses. Like other herpesviruses, EBV virions have a double-stranded, linear DNA genome. A protein tegument lies between the capsid and the envelope, which is embedded with glycoproteins that are important for cell tropism, host range, and receptor recognition [9]. Mature virions are approximately 120-180 nm in diameter [10,11]. The EBV genome of approximately 100 genes has been described in detail [8].

### Primary Infection and Lytic Replication

Initial infection is thought to occur in the oral (tonsillar) compartment (see Fig 1). The host cells of EBV are mainly lymphocytes and epithelial cells [9]. EBV attaches to B cells via binding of the viral gp350 protein to CD21 on B cells [12]. EBV gp42 then interacts with B cell human leukocyte antigen (HLA) Class II molecules and triggers fusion with the host membrane. In epithelial cells, which lack CD21, the EBV BMRF-2

protein interacts with β1 integrins [13-15], and the EBV gH/gL envelope protein triggers fusion via interaction with αVβ6/8 integrins [16]. Endocytosis of the virus into vesicles and fusion of the virus with the vesicle membrane releases the nucleocapsid into the cytoplasm. Once the viral nucleocapsid is dissolved, the genome is transported to the nucleus where it is replicated by DNA polymerases. Viral DNA polymerase accomplishes linear viral replication, which occurs during the lytic phase of the viral lifecycle.

Tsurumi et al. [7] have published a complete review of lytic and latent replication. Briefly, there are three temporal classes of viral lytic gene products (immediate early [IE], early [E], and late [L]). BZLF1 and BRLF1 are some of the IE products that further act as transactivators of the viral lytic program [7]. Activation of lytic replication or reactivation from latency is key to transmission. The early products (e.g., BNLF2a) have a wide array of functions including replication, metabolism, and blockade of antigen processing while late products tend to code for structural proteins such as the viral capsid antigens and gene products used for immune evasion (e.g., BCRF1).

An important consequence of EBV infection in B cells is to activate their growth program and trigger differentiation into a memory B cell via the germinal center reaction. Infected memory B cells are released into the peripheral circulation (see Fig 1), resulting in detectable levels of virus in the blood as discussed below. The number of infected B cells decreases over time after the onset of symptoms of primary infection [17], but these cells are never entirely eliminated.

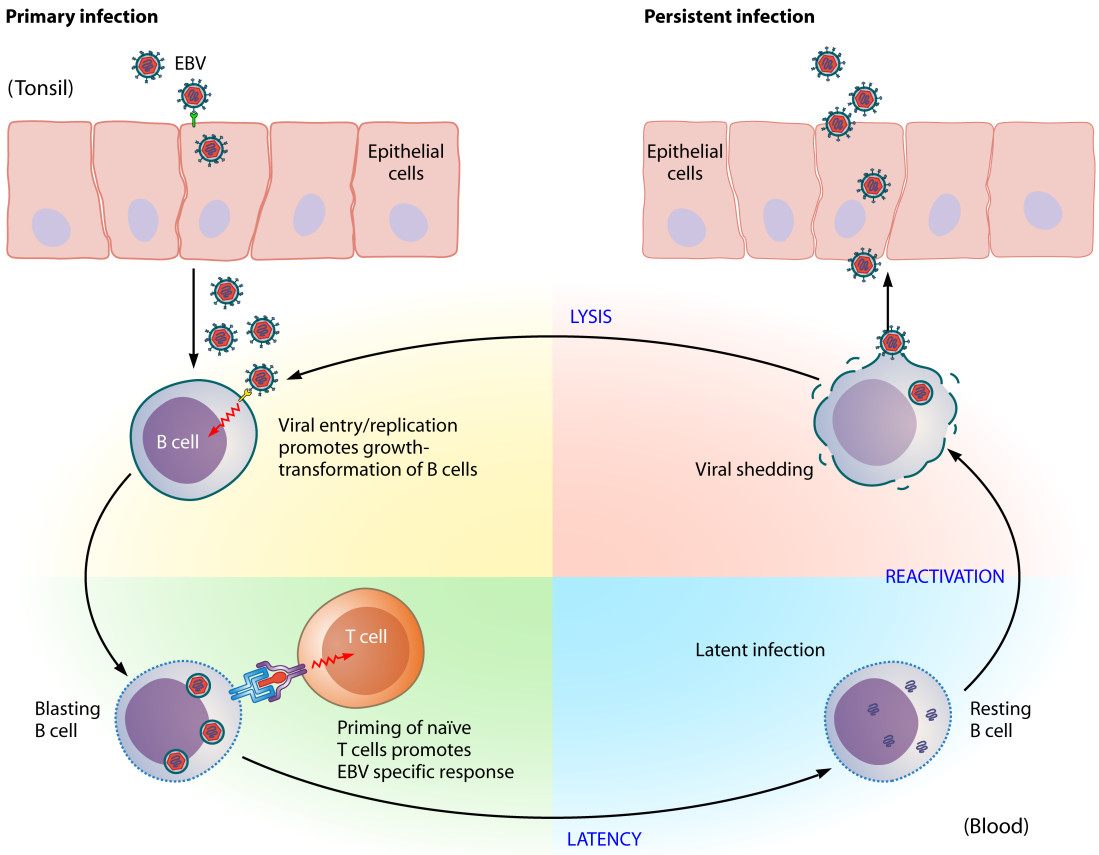


**Figure 1-1: EBV infection in normal carriers.** Primary EBV infection begins in the oral cavity. EBV uses different glycoproteins to infect epithelial cells and naïve B cells (i.e. CD21 on B cell depicted here in yellow). Viral entry results in transport of the EBV genome into the B cell nucleus where replication by cellular and viral DNA polymerases begins. EBV gene products activate the B cell growth program resulting in the proliferation of blasting B cells. Priming of naïve T cells by antigen presenting cells occurs in parallel. Normally, these blasting B cells are destroyed by cytotoxic T lymphocytes. Once in the circulation, previously activated memory B cells may continue to undergo lytic replication or, if EBV shuts down most of its protein encoding genes, latency occurs. At a later time, as cells recirculate between the oral and peripheral compartments, resting B cells may be activated resulting in viral reactivation and shedding.

**Latency**

Latency is the state of persistent viral infection without active viral production. EBV persists mostly in the memory B cell compartment and possibly also in epithelial cells [18] (see Fig. 1). Currently, it is thought that one in a million B cells carry the EBV genome in an individual after recovery from acute infection [5]. It is generally thought that EBV genomes in latently-infected B cells exist as episomes [19] although it is possible that the genomes exist as integrated DNA [11,20].

In contrast to lytic replication, episomal replication during the latent phase occurs via host DNA polymerase. There is limited expression of EBNA and latent membrane protein (LMP) gene products during latency [21]. These include EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA leader protein (EBNA-LP), LMP1, and LMP2. Characterization of gene expression patterns in different cell lines (i.e., Burkitt's tumors, EBV immortalized lymphoblastoid cell lines (LCL)) has determined that there are at least three different latency programs [18]. By using different transcription programs, EBV latent genomes can multiply in dividing memory cells (type I), induce B cell differentiation (type II), activate naïve B cells (type III) or completely restrict all gene expression in a context specific manner [18,22].

Only EBNA1 is expressed in the type I latency program, which is seen in Burkitt's lymphoma. CD8 T cells specific for many EBV antigens arise during the immune response to natural infection, however not for EBNA1, which contributes to evasion during latency [23]. EBNA1 and LMP1/2A are expressed in the type II latency program, which is observed in nasopharyngeal carcinoma and Hodgkin's lymphoma. LMP1 and LMP2 are responsible for B cell activation and induction of a growth (proliferation) program [5]. The type III latency program, in which all of the latency gene products are expressed, is often detected during acute infectious mononucleosis or in certain

immunocompromised individuals. Multiple factors that regulate gene expression in latency have been documented [22].

**Reactivation**

Latently infected B cells can occasionally be stimulated to reactivate EBV. This produces virus that can reinfect new B cells and epithelial cells, becoming a source of viral transmission (see Fig. 1). Although much is known about the molecular pathways involved in viral reactivation [24], what triggers reactivation *in vivo* is not known precisely. The presumption is that it occurs when latently infected B cells respond to unrelated infections because B cell receptor stimulation triggers reactivation in B cell lines. It is also not known what fraction of EBV infected cells are in the lytic or latent phase at any point in time, although a technique using serum from EBV-infected individuals may prove useful in the future [25]. Understanding how each gene product, whether lytic or latent, contributes to the pathogenesis of EBV-related diseases should lead to more rational and effective prevention and treatment strategies.

**EBV EPIDEMIOLOGY**

Young children acquire primary EBV infection most likely from close contact that involves exchange of oral secretions via shared items such as toys, bottles, and utensils. Before the age of 10, primary infection is usually asymptomatic or produces an acute illness that is often not recognized to be due to EBV [26]. In adolescents and young adults, however, primary EBV infection frequently presents as infectious mononucleosis, a clinical syndrome named by Sprunt and Evans in 1920 and discussed further below [27]. Hoagland argued convincingly that infectious mononucleosis was acquired “chiefly by direct intimate oral contact which allows for salivary exchange” [28]. His hypothesis was

strengthened by the lack of transmission among roommates [29-31] and failure to infect experimental volunteers [32]. He was also able to estimate its incubation period thanks to a patient who described a one-time, intense kissing experience 47 days before the onset of infectious mononucleosis. Hoagland questioned 73 subsequent patients, and 71 (97%) of them reported oral contact 32 to 49 days before developing infectious mononucleosis, which corresponds to an incubation period of approximately 6 weeks. In addition, an incubation period of 38 days has been reported for a well-documented case, which is consistent with Hoagland's observations [33].

Aside from oral transmission, EBV has been acquired from blood [34], indicating that virus present in the peripheral circulation, most likely in memory B cells [17], is or may become infectious. EBV can also be acquired from transplanted hematopoietic cells [35,36] or solid organs [37] and such infections can be life-threatening especially among recipients who were EBV-naïve before transplantation [38]. Several reports of intrauterine transmission of EBV have been published but none has been substantiated by appropriate viral studies [39,40]. Scottish investigators believe that EBV may be transmitted via genital secretions during penetrative sexual intercourse [41,42]. However, their data are retrospective and based on only 2 questionnaires completed 3 years apart. Furthermore, because kissing and sexual intercourse are virtually inseparable, oral transmission certainly cannot be ruled out.

The seroprevalence of EBV varies widely by geographic location [31,43-45]. Data indicate that primary EBV infection occurs at a younger age among persons from lower versus higher socioeconomic backgrounds [46,47], which has been attributed to crowded living conditions [26]. Acquisition of EBV by young children indicates that it can be transmitted without deep kissing. However, this does not rule out saliva as the source of EBV because young children commonly share objects that they put in their mouths.

Healthy people continue to shed EBV for many months after their acute infection and are potentially capable of transmitting it [48,49]. Because such virus "donors" are asymptomatic and hence not considered to be the source of infection, they often go unrecognized. For the most part, shedding becomes intermittent rather than continuous several months after the primary infection. Hadinoto et al. recently reported that EBV is shed continuously in the saliva at relatively stable levels over short periods (hours or days) but quantities varied as much as 4 to 5 $\log_{10}$ copies over months or years [17]. This suggests that a person's likelihood of transmitting EBV fluctuates over time.

No clear-cut seasonal pattern for infectious mononucleosis has been recognized [50,51]. However, more cases have been documented among U.S. college and university students when school is in session than during semester breaks [50] and Leard observed over a 12-year period that there was an October peak in admissions for infectious mononucleosis to the Boston University infirmary [52]. A peak incidence during June-August was reported among patients in the Israeli Defense Force [53], which the authors ascribed to more socializing of young people during the summer.

## PRIMARY EBV INFECTION

### Infectious Mononucleosis

Infectious mononucleosis was the name chosen by Sprunt and Evans to describe a syndrome that resembled an acute infectious disease accompanied by atypical large peripheral blood lymphocytes [27]. We now understand that these atypical lymphocytes, also called Downey cells, are activated CD8 T lymphocytes, most of which are probably responding to EBV-infected B cells.

Infectious mononucleosis most often begins insidiously with vague malaise followed several days later by fever, sore throat, swollen posterior cervical lymph nodes, and fatigue. Some patients experience an abrupt influenza-like onset with fever, chills, body aches, and sore throat. Table 1 displays the relative frequency of signs and symptoms compiled by combining data from other investigators [53-57] and our own experience [48,58,59]. It should be emphasized that the diagnosis of infectious mononucleosis cannot be made on clinical grounds alone.

Hepatitis, documented by abnormal liver function tests, is seen in 80% of cases and thus should be considered part of the acute disease rather than a complication. Liver involvement is subclinical in 90-95% of patients but the remainder develop jaundice and a few of them complain of tenderness in the right upper quadrant of the abdomen that is likely due to hepatic swelling with pressure on the liver capsule. Eyelid edema, which gives the patient a slit-eyed appearance and may be accompanied by facial puffiness, is a useful clinical clue if present because it is unique to primary EBV infection [60].

**TABLE 1-1. Prevalence of signs, symptoms and laboratory abnormalities in infectious mononucleosis**

| Finding | Prevalence (%) | Comment |
|---|---|---|
| Signs | | |
| Pharyngitis | 100 | Occasionally seen without sore throat |
| Cervical lymphadenopathy | 95 | Especially posterior cervical and postauricular |
| Fever | 50 | Often masked by antipyretics |
| Hepatomegaly | 25 | |
| Splenomegaly | 33 | |
| Eyelid edema | 10 | Unusual in other acute illnesses |
| Rash | 5 | Virtually all patients given penicillin derivatives develop a rash |
| Symptoms | | |
| Sore throat | 95 | Many patients describe this as the "worst" they have ever had |
| Fatigue | 90 | Usually the last symptom to resolve |
| Headache | 75 | Common but underappreciated |
| Feverish | 70 | |
| Body aches | 50 | Patients describe this as "like the flu" |
| Decreased appetite | 50 | |
| Abdominal discomfort | 40 | Due to mesenteric adenitis or hepatosplenomegaly |
| Laboratory Abnormalities | | |
| ALT elevation | 80 | 5-10% of patients are jaundiced |
| Leukocystosis | 40 | Usually due to increase in CD8 cytotoxic lymphocytes |
| Thrombocytopenia | 25 | Thought to be autoimmune |
| Anemia | 10 | Thought to be autoimmune |
| Median duration of illness | 16 days | Mean, 19 days |

Based on a compilation of published series [53-57] and 116 subjects followed in natural history and treatment trials at the University of Minnesota [48,58,59].

Recovery is gradual and it may take months for the patient to feel entirely well [57]. Fatigue interferes with productivity and quality of life and is usually the last symptom to resolve. Recrudescence of symptoms before the acute illness ends occurs occasionally [56]. However, recurrences or "second cases" of infectious mononucleosis documented by laboratory evidence of active EBV infection after recovery from the acute illness are very uncommon. Hoagland reported no recurrences in his series of 200 patients, most of

whom were hospitalized during their acute illness according to military policy [55]. We have had just one laboratory-documented recurrence among 116 subjects who acquired infectious mononucleosis between the ages of 16 to 26 years (H. H. Balfour, Jr., unpublished data).

The risk of developing infectious mononucleosis after primary EBV infection correlates with the age of the patient [51]. Children younger than 10 are usually asymptomatic or moderately ill with a partial infectious mononucleosis syndrome, although classic infectious mononucleosis can occur in this age group [61]. Primary EBV infection among adolescents and young adults may also be asymptomatic but at least half of them develop full-blown infectious mononucleosis (H. H. Balfour, Jr., unpublished data). The reason for this age-specific severity of illness remains elusive. The severity of primary EBV infection in adults increases with age and patients older than 40 are especially prone to serious illness [62,63]. They have more prolonged fever and more serious hepatic involvement but less noticeable lymphadenopathy than younger patients.

Many complications have been associated with infectious mononucleosis but nearly all of them are uncommon or rare [64-66]. Table 2 lists the complications whose frequency is estimated to be at least 1%. The following complications, listed alphabetically, have been described in fewer than 1% of patients: conjunctivitis, hemophagocytic syndrome, myocarditis, neurologic diseases other than meningoencephalitis, parotitis, pancreatitis, pneumonitis, pericarditis, psychological disorders, and splenic rupture [64-68]. The latter is a rare but greatly feared complication that excludes athletes from contact sports for variable periods of time (see the section on Limitation of Activities below).

**TABLE 1-2. Complications reported in ≥1% of cases of infectious mononucleosis**

| Complication | Comment |
|---|---|
| Airway obstruction | Due to oropharyngeal swelling and edema |
| Meningoencephalitis | Other neurologic complications have been reported but are rare |
| Hemolytic anemia | Thought to be autoimmune |
| Thromobocytopenia | Thought to be autoimmune |
| Rash | Rash due to EBV is uncommon, but maculopapular rashes occur in the majority of patients inadvertently given penicillin derivatives |

### Asymptomatic or Unrecognized Primary EBV Infections

As stated above, EBV infections in children under the age of 10 are often overlooked, either because they are entirely asymptomatic or do not present with a typical infectious mononucleosis syndrome. A clinical dilemma for making the correct diagnosis in children is that point-of-care laboratory tests, which are essentially all heterophile antibody assays, may be falsely negative [61,69]. Hence, even suspected EBV infections in children may not be confirmed. Primary EBV infection may not be recognized in adolescents and young adults either, but 90% of them report some symptoms especially sore throat if seen shortly after the onset of infection (H. H. Balfour, Jr., unpublished data) and could be diagnosed with appropriate laboratory tests.

## DIAGNOSIS OF EBV INFECTION

Primary EBV infection can be diagnosed with certainty only by utilizing the appropriate laboratory tests. Patients who are mildly ill are unlikely to be identified because they either do not seek medical attention or EBV infection is not considered in the differential diagnosis. Patients with a typical infectious mononucleosis syndrome (described above) are still a diagnostic challenge because their signs and symptoms are not very sensitive or specific for EBV infection. For example, a recent report found that

the classic triad of fever, sore throat, and lymphadenopathy had a sensitivity of 68.2% and a specificity of 41.9% for EBV infection [53].

Several signs and symptoms point to an EBV etiology (Table 1). These include a very sore throat that appears inflamed, swollen, and sometimes has a membranous exudate, symmetrical posterior cervical and postauricular lymphadenopathy, and eyelid edema often accompanied by facial puffiness. Clinical findings that militate against EBV infection are rhinorrhea, cough, and rash, unless the patient is taking ß-lactam antibiotics, in which case the rash is due to transient hypersensitivity to penicillin derivatives induced by EBV [70,71].

**Nonspecific Laboratory Tests**

Nonspecific laboratory test that hint at infectious mononucleosis include a peripheral blood smear, heterophile antibodies and liver function test. In 1920, Sprunt and Evans reported 6 young adults with very similar acute infections who had "a mononuclear leucocytosis instead of the more usual increase in the polymorphonuclear leucocytes [27]." They illustrated the features of these mononuclear cells that distinguished them from leukemia. Several years later, Downey and McKinlay published a comprehensive description of the atypical lymphocytes seen in the peripheral blood of patients with infectious mononucleosis [72]. As mentioned above, these atypical lymphocytes, also referred to as Downey cells, are activated CD8 T lymphocytes, most of which are thought to be responding to EBV-infected B cells. While they are invariably present in primary EBV infection, they may also be found in infectious mononucleosis-like illnesses due to other viruses, especially cytomegalovirus [73].

In 1932, Paul and Bunnell discovered that heterophile antibodies, specifically sheep cell agglutinins, were elevated during acute infectious mononucleosis but not in many other diseases and thus could be used for diagnosis [74]. They defined heterophile

antibodies as “having the capacity to react to certain antigens, which are quite different from, and phylogenetically unrelated to the one instrumental in producing the antibody response” [74]. Heterophile tests use various mammalian erythrocytes to detect IgM class antibodies, which are present during the generalized immune upregulation that characterizes acute primary EBV infection.

Heterophile antibodies detected using bovine erythrocytes were present in 76 (96%) of 79 adults during the first 10 days of laboratory-confirmed EBV infectious mononucleosis (H. H. Balfour, Jr. unpublished data). Hence, heterophile tests are a sensitive diagnostic method for acute infectious mononucleosis. The laboratory diagnosis of infectious mononucleosis is now almost always made by a heterophile antibody test [75]. However, they do have drawbacks. Approximately 40% of children 4 years of age or younger do not develop heterophile antibodies during primary EBV infection [69]. Thus, the heterophile test may be falsely negative in young children. Secondly, heterophile antibodies are nonspecific and may be present in non-EBV infections, malignancies and autoimmune diseases [76,77]. Finally, heterophile antibodies may persist for a year or more and therefore do not always signify an acute EBV infection [78].

On average, 80% of patients with infectious mononucleosis have abnormal liver function during the early stages of infection (Table 1). Elevated liver enzymes, especially alanine aminotransferase, strengthen the clinical impression of infectious mononucleosis.

**EBV-specific Assays**

Indirect immunofluorescence or EIAs are the common platforms for the detection of EBV-specific antibodies. As discussed above and illustrated by Fig. 2, the profile of EIA antibodies present distinguishes acute primary, convalescent, and past infection.

Acute primary EBV infection is characterized by IgM antibodies to the early antigen VCA in the absence of IgG antibodies to the latent antigen EBNA1. VCA IgG antibodies may be present in acute infection, but in lower quantity than VCA IgM antibodies. During convalescence (from the third week to the third month after onset of illness), VCA IgM antibodies dwindle while VCA IgG antibodies rise and persist for life. Between the third and sixth month, VCA IgM antibodies disappear whereas EBNA1 IgG antibodies become detectable and persist for life. All 3 antibodies may be present in late primary infection or subclinical reactivation, which can be distinguished from each other by performing an IgG avidity assay [79]. An evidence-based correlation of serologic patterns with stages of EBV infection has recently been published [80]. This analysis included heterophile antibody and EA-D IgG antibody in addition to VCA IgM and IgG, and EBNA1 IgG.

In addition, there are specific test that can detect and quantify EBV. EBV can be identified in tissue samples by immunohistochemical approaches. In situ hybridization to detect EBV-encoded RNA transcripts (EBERs) is the gold standard for detecting EBV in tissue [81]. PCR is the technique of choice for detecting and quantifying EBV in body fluids and can also be used to quantify the virus in tissue samples [82]. While there are options in terms of platforms, volumes, probes, and targets, a multicenter comparison of different real-time PCR assays suggested that if samples are tested at one center on the same platform, real-time PCR is a precise technique for measuring viral load [83]. However, substantial quantitative differences were found when samples were tested in different laboratories. This is because the gene targets and assay platforms differ widely among laboratories and at present there is no universal quantitative standardization.

The best matrix to use for monitoring EBV infections is debated. Because most assays employ a DNA template and the matrix tested is usually blood, EBV blood viral loads are also referred to as EBV DNAemia. However, some experts recommend

plasma [84] but the majority favor whole blood [85,86]. Copy numbers in plasma are usually 10 to 100-fold lower than in concomitant whole blood samples. Immunocompetent patients with symptomatic EBV infections have viral loads averaging 5,000 copies/mL of whole blood during the first 7 to10 days of illness as compared with levels of 5,000 to ≥50,000 copies/mL of whole blood in transplant recipients; viral loads during latency are rarely >1,000 copies/mL of whole blood (H. H. Balfour, Jr., unpublished data).

Quantitative PCR is also useful for monitoring the effects of anti-EBV therapy and in the evaluation of candidate antiviral drugs [58]. As mentioned above, when used to evaluate the response to therapy, the goal is to drive the blood viral load below the level of detection or at least to <1000 copies/mL. In addition, quantitative PCR may be useful for making the correct viral diagnosis in patients with atypical clinical features or in young children who have heterophile-negative infectious mononucleosis [87].

## PRIMARY RESPONSE TO EBV INFECTION

A potent innate and adaptive immune response occurs during primary EBV infection. This response, although it controls infection, does not eliminate it and the virus persists for the lifetime of the infected individual. Thus, a careful balance between the virus and the immune system exists. The section here will summarize what we have learned from virological and immunological studies.

### Virologic Events

Infectious virus acquired by exposure to oral secretions or blood establishes a foothold in the oral and/or blood compartment in B lymphocytes, epithelial cells, or both [88,89]. After that, it takes an estimated 5 to 7 weeks for the primary EBV infection to manifest itself as infectious mononucleosis. Computer simulations based on a scenario

of multiple rounds of viral replication, with amplification at each step, can predict peak viral loads consistent with that observed in natural infection [90]. Unfortunately, there are few data on immune or virologic events from samples during the incubation period, as patients do not seek medical care prior to the onset of symptoms. Thus, what happens during the incubation period remains a major unanswered question about primary EBV infection. Our studies discussed in Chapter 2 provide some insight into these events.

Because the onset of infectious mononucleosis is often insidious, little is known about the virus-host interactions until about the fifth day of illness at the earliest [48,58]. Viral loads in the oral cavity, especially in oral cells, are on average 1-2 $\log_{10}$ EBV copies/mL higher than in whole blood. As shown in Fig. 2, viral clearance from the oral compartment is much slower than from the blood. Viral loads in oral cells and saliva remain elevated for many months [17,48,49,58]. In contrast, virus is eliminated from whole blood more rapidly [48]. Detectable virus in the blood is eliminated by the seventh week of illness in almost every patient, but it did recur 6 months or more after primary EBV infection in 8 (10%) of 78 subjects we have been following for several years (H. H. Balfour, Jr., unpublished data*).*

Viral DNA detected in the blood is thought to come primarily from infected memory B cells [18,91]. It was previously held that EBV only maintains latency in B lymphocytes, based in part on the fact that infection was eradicated by an EBV negative bone marrow transplant [92]. However, viral gene expression patterns differ when the virus emerges from epithelial cells versus B cells in a way that suggests passage back and forth [93]. Furthermore, patients depleted of B lymphocytes with anti-CD20 antibody, rituximab, still shed virus from the throat [94], suggesting that there could be a reservoir of EBV latency other than the B cell.

One gap in our understanding the implications of viral load on the epidemiology and pathogenesis of infectious mononucleosis is not knowing what portion of the viral

load is complete and potentially infectious, and what portion is either in episomal or unencapsidated form. The latter could certainly be immunogenic but would neither be contagious nor cause tissue-invasive disease [19].

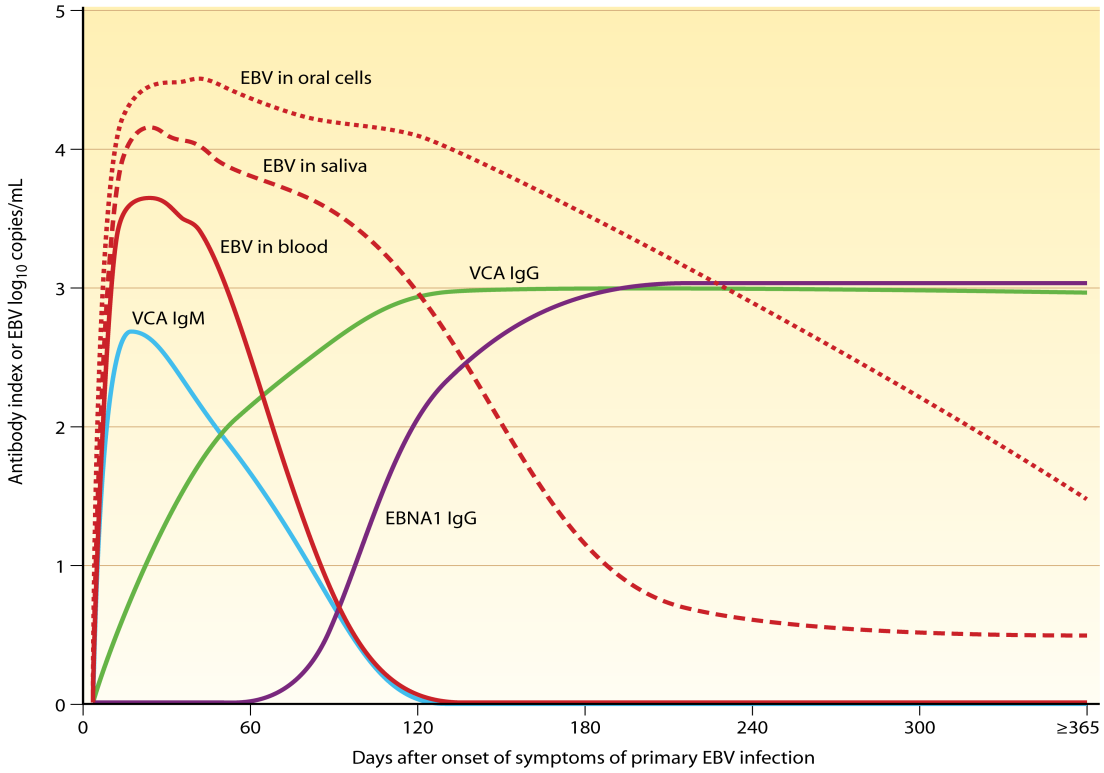


**Figure 1-2: Kinetics of the EBV-specific antibodies and viral load in infectious mononucleosis**. At presentation, EBV may not be detected in the blood but is usually found in large quantities in the oral cavity. Virus is cleared from the blood much more rapidly than from the oral compartment. Oral viral shedding can persist for months and recurs intermittently for years in most healthy adults. At the onset of illness, most patients have IgM antibodies to EBV VCA; these decline between 2 to 6 months after infection. VCA IgG antibodies may be detected as early as the first 2 weeks of illness. Essentially 100% of patients have detectable VCA IgG antibodies during convalescence and these persist for life. EBNA1 IgG antibodies do not develop until 3 to 6 months after infection but then persist for life.

## Immune Response to EBV

**Innate immune response.** The innate immune system is an important first line of defense against viral infections. Viruses elicit a strong type I interferon (IFN) response early after infection. This is presumed to be the case for primary EBV infection, although as alluded to above, the kinetics and quality of this response are difficult to study *in vivo* because of the long incubation period. Nonetheless, EBV potently stimulates IFN production from isolated human plasmacytoid dendritic cells *in vitro* [95]. Viral DNA and protein are recognized by pattern recognition receptors (PAMPs), such as toll-like receptors (TLR), which can trigger an IFN response, facilitate the activation of natural killer cells, and act in multiple ways to prime the adaptive immune response. Our laboratory has recently defined the transcriptional profile of human blood during primary EBV, and both type I and type II interferon regulated genes were strongly upregulated (K. A. Hogquist, unpublished data). There is evidence for the involvement of multiple TLR in activating the innate response to EBV, including TLR2 [96], TLR3 [97], TLR7, and TLR9 [95]. Interestingly, the virus may also have mechanisms of controlling TLR signaling [98].

The inflammatory cytokines TNF$\alpha$, IL-6, and IL-1ß were increased in tonsillar tissue from patients with infectious mononucleosis [99]. Many studies have detected inflammatory cytokines in the serum of individuals with infectious mononucleosis as well (Table 3). Prominent amongst these is IFN$\gamma$. IFN$\gamma$ is produced by activated T cells and NK cells. Not only is IFN$\gamma$ itself elevated, the catabolic product neopterin, which is produced by monocytes that are stimulated with IFN$\gamma$, is also elevated (Table 3). IFN$\gamma$ is thought to be important for control of EBV infection and reactivation, based on studies of a related *gammaherpesvirus* infection in mice [100-102]. However, high levels of IFN$\gamma$ likely

contribute to the symptoms experienced during infectious mononucleosis, as this cytokine is known to cause headache, fatigue, and fever [103].

Interestingly, type I interferon (IFN$\alpha$) is not consistently detected in the serum of infectious mononucleosis patients [104-106]. This may reflect both the fact that IFN$\alpha$ can be difficult to detect, and that it is more likely to be produced early in the response to viral infection, before the onset of infectious mononucleosis symptoms and presentation in the clinic. The inflammatory cytokines TNF$\alpha$ and IL-6 are also elevated during acute infectious mononucleosis. Finally, serum IL-2 is elevated during infectious mononucleosis consistent with the dramatic expansion of CD8 T cells.

**TABLE 1-3. Alterations in serum cytokine levels during infectious mononucleosis**

| Serum Cytokines Observed | Status during infectious mononucleosis | Possible Impact on Clinical Pathogenesis (in terms of known function) | Ref |
|---|---|---|---|
| Interferon-gamma (IFNγ) | Elevated | Type II interferon, produced by NK cells, and Th1 and CD8 T cells. Broad immunostimulatory effects. Important for control of chronic infection. Likely inhibits viral replication and reactivation. | 104,106-110 |
| Neopterin | Elevated | A pteridine compound released from macrophages/monocytes stimulated by IFNγ. | 104,107,110 |
| Interferon-alpha (IFNα) | Not reproducibly detected | Type I interferon, produced by monocytes and plasmacytoid DC. Broad anti-viral and immunostimulatory effects. Important for control of acute infection. | 104-106,109 |
| Interleukin-6 (IL-6) | Elevated | Inflammatory cytokine produced by T cells and macrophages. Mediator of fever and acute phase response. Promotes B cell maturation. | 104,109-111 |
| Tumor Necrosis Factor alpha (TNFα) | Elevated | Inflammatory cytokine produced mainly by macrophages. Activates macrophages, stimulates acute phase response, and can cause liver dysfunction, fever. | 107,111 |
| Interleukin-12 (IL-12) | Elevated | Cytokine produced by dendritic cells. Promotes differentiation of Th1 CD4 and CD8 T cells. Enhances NK and CTL cytotoxicity. | 106,108 |
| Interleukin 2 (IL-2) | Occasionally elevated | Produced by activated T cells. Growth factor for regulatory T cells. | 106-109,111 |
| Interleukin-10 (IL-10) | Elevated | Immunosuppressive cytokine produced by monocytes and T cells. In combination with viral IL-10, may suppress T cell production of other cytokines (IFNγ, TNFα) and enable systemic spread of virus. | 111-113 |
| Transforming Growth Factor beta (TGFβ) | Elevated | Immunosuppressive cytokine with pleiotropic effects. | 113 |

The immunosuppressive cytokines, IL-10 and TGFß, are also detected in the serum of infectious mononucleosis patients (Table 3). Interestingly, the EBV late gene BCRF1 acts as an interleukin-10 (IL-10) homologue and shares 84% of its amino acid sequence with human IL-10 [114]. During acute infectious mononucleosis, both viral and host IL-10 are detected in sera [112]. Host IL-10 is produced by monocytes and lymphocytes, and functions to suppress T cell proliferation and cytokine production, and can inhibit IFNγ production from T cells [115]. Thus it might be predicted that IL-10 counters the pathogenic effects of IFNγ during infectious mononucleosis. Consistent with this, the highest levels of IL-10 were observed in patients with a shorter duration of symptoms [113]. Furthermore, high levels of IL-10 are observed in PTLD patients, and are reduced as PTLD resolves with an effective antiviral response (reviewed in [116]). Thus overall, it would appear that IL-10 and IFNγ play key roles in the balance of immune protection and symptoms during infectious mononucleosis.

NK cells are another important component of the immune response and are thought to play a key role in regulating chronic viral infections [117]. In fact, human NK cell deficiencies are associated with increased susceptibility to several viral (and bacterial) infections including EBV [118]. NK cell numbers increase during infectious mononucleosis [106,119,120]. Interestingly, their numbers were inversely associated with disease severity [106], suggesting that NK cells could play a role in limiting viral replication.

**Adaptive immune response**. The adaptive immune response to EBV has been extensively studied, and was discussed in detail in a recent review [121]. Both humoral and cellular immune responses are generated. The humoral or antibody response is critical in diagnosing infectious mononucleosis, and the cellular response (particularly the CD8 T cell response) is critical for controlling viral replication, but may also contribute to the severe symptoms of infectious mononucleosis.

The kinetics of specific antibody responses to primary EBV infection as measured by enzyme immunoassay (EIA) are shown in Fig. 2. The first humoral response detected is an IgM class antibody directed against the viral capsid antigen (anti-VCA IgM). This antibody was present all 70 of our subjects with primary EBV infection who were tested. Sixty-three (90%) of them were positive within 7 days after the onset of symptoms, six became positive during the second week of illness, and one did not become positive until 49 days after the onset of illness (H. H. Balfour, Jr., unpublished data). All patients develop anti-VCA IgG antibodies, which peak during the first 2-4 months and then persist for life. IgG antibodies to the latent antigen, EBNA1, do not develop in most individuals for about 3 months but once they appear, they persist for life [122]. Antibodies to the early antigen diffuse (EA-D) are also elicited during acute infection in 60-80% of patients, but they are not diagnostic of a specific phase of EBV infection and hence are not generally useful [123].

Anti-gp350 antibodies may be detected after natural exposure to EBV or in response to gp350 subunit vaccines (discussed below) [124-127]. Furthermore, it has been suggested by Turk et al. that gp350 antibodies enhance epithelial cell infection [128]. Interestingly, this could suggest another form of immune evasion, this time from neutralizing antibodies of the humoral arm, that allows EBV to be maintained in a

alternate reservoir (tonsillar epithelium) when its initial reservoir (B cells) are being depleted by an active immune response (T cells).

Both CD4 and CD8 T cells make a robust response to EBV antigens, and over 50 HLA Class I and Class II epitopes from this virus have been identified [121]. Early in infection, CD8 T cells specific for lytic antigens tend to dominate the response, while CD4 and CD8 T cells specific for latent antigens do not show such a large burst, but persist for life [129]. The massive lymphocytosis in the blood that characterizes infectious mononucleosis is thought to consist largely of CD8 T cells specific for EBV lytic antigens [121], although possible activation of bystander (non-EBV specific T cells) has not been rigorously ruled out. We address this in Chapter 3.

Interestingly, this large adaptive immune response is thought to be responsible for the major symptoms of infectious mononucleosis, as disease severity correlated more closely with lymphocytosis than viral load in a small study [130]. Additionally, EBV-specific CD8 T cells were found to be under-represented in tonsil compared with blood early during infection. This resolved later, suggesting that efficient control of EBV infection requires tonsillar homing of CD8 T cells[131]. Ultimately, CD8 T cells are critical for control of EBV, as evidenced by the occurrence of EBV lymphoproliferation and lymphomagenesis in immunosuppressed patients [132] and the efficacy of EBV specific CD8 T cell transfers in controlling PTLD [133].

**Pathogenesis of Infectious Mononucleosis**

As mentioned above, the robust adaptive immune response is thought to be responsible for the major symptoms of infectious mononucleosis. But the question remains, why is the infectious mononucleosis syndrome during primary EBV more common in adults than children? It was proposed that adults through sexual activity

acquire a higher viral dose than children through salivary contact [134]. This higher viral dose would initiate a larger CD8 T cell response, which would cause the symptoms of infectious mononucleosis through production of inflammatory cytokines. However, at least one study found that symptomatic infectious mononucleosis was not associated with a higher viral load [130]. Others have speculated that pre-existing immunity to other viruses, which cross react with EBV (called "heterologous immunity") could provide a robust CD8 T cell response to primary EBV [135]. This could explain why adolescents and adults tend to experience infectious mononucleosis, while children are largely asymptomatic, as adults are likely to have broader immune experience in general. However, neither the subunit or peptide vaccine studies (discussed below) suggested that pre-existing immunity to EBV causes more severe infectious mononucleosis (in fact the reverse was observed). Thus, it is unclear how heterologous immunity would impact the primary response to EBV.

Another possibility relates to the innate immune response. As mentioned above, elevated NK cell numbers were shown to correlate with reduced disease severity in infectious mononucleosis [106]. It was recently shown in a mouse model of chronic viral infection (murine cytomegalovirus) that NK cell mediated lysis of infected dendritic cells limited the CD4 and CD8 T cell response and, paradoxically, resulted in viral persistence [136]. If the large CD8 T cell response is responsible for disease severity during infectious mononucleosis, then NK cells may reduce it by limiting the adaptive immune response. Thus, in Chapter 2 of this thesis, we asked if NK cell increases are associated with lower CD8 T cell responses?

Finally, high levels of inflammatory cytokines could also be responsible for the symptoms observed during acute infectious mononucleosis, produced by either innate or adaptive immune cells. Therefore, it is important that future studies examine multiple

parameters to better understand the factor(s) that mediate pathogenesis. This thesis lays the foundation.

## TREATMENT

### Symptomatic Management of Infectious Mononucleosis

**Antipyretics.** Most clinicians favor acetaminophen over aspirin to control fever, because of concern that aspirin might increase the risk of hemorrhage into the spleen [137-139]. Fever usually subsides within a week but has been reported to persist for up to 3 weeks [55].

**Analgesics.** Pain control is important during the early stages of infectious mononucleosis especially for those patients whose throat is so sore that it keeps them up at night. Recommended pain management includes acetaminophen, nonsteroidal anti-inflammatory drugs, salt-water gargles, anesthetic throat lozenges, or viscous lidocaine hydrochloride [137-139]. Codeine sulfate can be prescribed for subjects who do not respond to non-narcotic pain control. However, codeine can cause constipation, in which case stool softeners or laxatives are advisable [137].

**Fluids and nutrition.** Attention to fluid intake is important especially for febrile patients. Maintaining adequate nutrition is also important but can be challenging because many patients are anorexic during the first week or two of illness and food is cloying.

**Limitation of activities.** Bed rest is unnecessary but contact sports are contraindicated [137-140]. Patients generally adjust daily activities to the level of their exercise tolerance. When may athletes return to contact sports? There is no consensus. A recent evidence-based review suggested that they may return as early as

3 weeks after the onset of illness provided they are afebrile, have no remaining clinical symptoms, and have normal energy [140].

**Corticosteroids.** A review of the medical records of Rochester Medical Center, Rochester, NY found that nearly 45% of patients with infectious mononucleosis received systemic corticosteroids [141]. Hence, the use of corticosteroids in infectious mononucleosis is a relatively common practice. However, an evidence-based literature review of 7 studies concluded that there is insufficient evidence to recommend steroids for control of the symptoms of infectious mononucleosis [142]. Most authors—and this is our practice also—reserve corticosteroids for management of complications, such as impending airway obstruction, autoimmune anemia, and autoimmune thrombocytopenia [137-140].

**Antiviral Drugs**

A number of antiviral drugs have *in vitro* activity against EBV [143-149]. Most of these drugs are nucleosides but a few non-nucleosides are also in the pipeline. Unfortunately, there is no standard formula for equating *in vitro* susceptibility with clinical efficacy. For many nucleosides including acyclovir and ganciclovir, plasma concentrations do not reflect the active antiviral drug moiety. Nucleoside analogues must first be taken up by virus-infected cells and phosphorylated to their active triphosphate derivatives, which then inhibit viral DNA synthesis [150]. Although intracellular nucleoside triphosphate concentrations are difficult to measure analytically, the area-under-curve (AUCs) and half-lives of these active metabolites will most likely have the best correlation with *in vivo* antiviral efficacy.

Nucleosides are the only class of antiviral drugs that has been evaluated for treatment of EBV infections in controlled clinical trials. These nucleosides are DNA

polymerase inhibitors. They are inactive as given but when anabolyzed intracellularly to the active nucleoside triphosphate form, they act as faulty substrates for viral DNA polymerase, disrupting or terminating synthesis of the DNA chain (reviewed in[151]). The monophosphorylation step is accomplished more efficiently by viral-encoded enzymes than by host cell nucleoside kinases, thus enhancing the activity of these compounds in herpesvirus-infected cells as compared with uninfected cells [150]. The enzyme responsible for monophosphorylation in EBV-infected cells appears to be a viral-encoded protein kinase rather than a thymidine kinase [152,153].

Acyclovir trials have uniformly shown a reduction of EBV in the oral compartment, but clinical efficacy was not demonstrated [154-158]. Subsequently, prodrug of acyclovir, valacyclovir, has been studied for clinical efficacy in EBV-related disease. Because valacyclovir has an oral bioavailability of at least 50% as compared with 10-20% for the parent compound [159], it has the potential to exert more potent *in vivo* anti-EBV activity.

Simon et al. [160] performed a 3-arm, placebo-controlled, double-blind trial of valacyclovir, valacyclovir with prednisolone, or placebo in children 2-18 years old with "EBV illness." Although this authors in this study did not substantiate the diagnosis of EBV infection, the data did suggest that the overall disease burden was lessened by valacyclovir with or without prednisolone as compared with placebo. In a no placebo arm study, Balfour et al. evaluated valacyclovir versus no antiviral drug in 20 University of Minnesota undergraduates with infectious mononucleosis due to laboratory-confirmed primary EBV infection [161]. In this group, the number of reported symptoms and the severity of illness were reduced significantly among the 10 valacyclovir recipients as compared with the 10 control subjects. In summary, the effect of valacyclovir or any other candidate anti-EBV drug on blood viral load is worthy of further investigation as

reduction in EBV DNAemia could be the antiviral effect most closely associated with clinical improvement.

### Management of Serious or Life-threatening EBV Disease

Potentially serious EBV disease in transplant patients is managed initially by a reduction of immunosuppression [38]. If that is insufficient to bring the viral infection under control, humanized monoclonal anti-CD20 (rituximab) may be administered sometimes in conjunction with chemotherapy [162]. In refractory cases, adoptive immunotherapy with primed CD8 + T cells [163,164] has sometimes been successful.

## PREVENTION

### Minimizing Exposure to EBV

As discussed above, EBV infections can be serious and even life-threatening in transplant recipients. Primary EBV infection after transplantation could be prevented, at least in part, by finding EBV-naïve (seronegative) donors for EBV-naïve recipients. However, because >90% of adults worldwide are seropositive, identifying a suitably matched seronegative donor is impractical. Even if an EBV-naïve donor could be found, the virus still might be acquired after transplantation by the natural route. A more practical approach would be to immunize transplant candidates several months before transplantation, which is discussed in the section on gp350 vaccine below.

### Antiviral Prophylaxis

Antiviral drugs (acyclovir, valacyclovir, ganiciclovir, or valganciclovir) are routinely given to patients for 3 to 6 months after transplantation to prevent or suppress herpesvirus infections with the major focus being on prevention of cytomegalovirus

(CMV) disease. Some transplant centers increase the antiviral drug to treatment dosage in asymptomatic patients with rising CMV DNAemia. This is called preemptive therapy. Whereas anti-herpes drugs clearly reduce the incidence and severity of posttransplant CMV disease [165,166], their role in the management of posttransplant EBV disease has not been established [167,168].

**Vaccines**

Development of a prophylactic vaccine, in our opinion, is the most important future step toward controlling the consequences of primary EBV infection. A prophylactic EBV vaccine was proposed by Epstein and Achong in 1973 [169] but several problems—real or perceived—have hindered its progress [170]. At long last, two very different adjuvanted EBV vaccines have been evaluated in placebo-controlled clinical trials [127,171]. One vaccine contains a gp350 subunit antigen and the other a $CD8^+$ T-cell peptide epitope.

**gp350 subunit vaccine.** The EBV envelope glycoprotein, gp350, (formerly known as gp340 or EBV-induced cell membrane antigen) has been considered to be an attractive immunogen ever since antibodies to gp350 were shown to neutralize the virus [172]. Despite a study showing that vaccine-induced anti-gp350 antibodies did not protect cottontop tamarins from developing tumors after a lymphomagenic challenge dose of EBV [173] work on a gp350 vaccine continued.

Gu et al. performed a Phase I vaccine trial in Beijing, China using vaccinia virus constructs expressing gp350 [124]. After the vaccine was shown to be immunogenic in seropositive children, EBV-naïve children were studied. The subjects were 1 to 3 years of age. Nine received the vaccine and 10 subjects served as controls. During 16 months of follow-up, 3 of 9 vaccinees were infected with EBV versus 10/10 in the control group.

The authors concluded: “it has been shown for the first time that protection against and/or delay of EBV infection by the natural route is possible.”

Several years later, successful production of a recombinant gp350 construct in Chinese hamster ovary cells was reported [174]. An adjuvanted EBV vaccine containing this antigen was subsequently employed in four clinical trials [125-127]. The first two evaluated safety and immunogenicity only [125]. The third was a placebo-controlled, double-blind study that also evaluated efficacy by following subjects for up to 19 months postimmunization for evidence of EBV infection [127]. The vaccine did not prevent infection: 13 (14%) of 90 vaccine recipients became infected versus 18 (20%) of 91 placebo subjects. However, it had a significant effect on clinical disease. Infectious mononucleosis developed in 2 (2%) of 90 vaccinees as compared with 9 (10%) of 91 placebo recipients. Failure to prevent EBV infection while preventing clinical illness might actually turn out to be an advantage for the vaccine, if the observation that latent infection by a murine counterpart of EBV (murine γ-herpesvirus 68) protected mice from subsequent bacterial infection applies to humans [175].

Finally, Rees et al. administered multiple doses of this vaccine to 16 pediatric renal transplant candidates [126]. The vaccine was well tolerated and all 13 evaluable subjects mounted an anti-gp350 antibody response. However, only four made a neutralizing antibody response. Because there was no control group, vaccine efficacy could not be assessed but this small Phase I trial did show that pretransplant immunization is feasible.

**$CD8^+$ T-cell peptide epitope vaccine.** Another strategy to control expansion of EBV infected B cells and prevent infectious mononucleosis is to generate CD8 T cell immunity to EBNAs [176]. The potential role of these viral proteins in B cell transformation precludes their use in whole protein based vaccines, thus a peptide vaccine was generated and tested. This trial utilized an EBNA3A peptide epitope (FLRGRAYGL)

restricted by HLA B8 [177] with tetanus toxoid (TT) formulated in a water-in-oil adjuvant as a source of T cell help [171]. EBV seronegative individuals were immunized on a two-month interval schedule. Of the fourteen enrolled subjects, four received placebo, two were immunized with 50 μg dose of peptide and the remaining 8 individuals were immunized with a 5 μg dose. This strategy was effective at generating a peptide specific CD8 response in most individuals as measured by *ex vivo* peptide specific IFNγ production. While this was a small study, no infectious mononucleosis occurred in 4 peptide-vaccinated subjects who subsequently acquired EBV infection, whereas it did occur in 1 of 2 subjects in the placebo group.

The general utility of epitope vaccines is limited by the fact that they only target specific HLA types. Nonetheless epitope vaccines might be useful for PTLD patients, where the HLA type is typically known. This trial was a also a "proof-of-principle" study, which showed that EBV vaccines that generate CD8 immunity are safe and did not exacerbate EBV immune responses after primary infection.

**SUMMARY OF PROGRESS AND PROBLEMS**

In this chapter, we have discussed the clinical, virologic, and immunologic features of primary EBV infection. The clinical consequences of primary infection have been well described for adults after the onset of infectious mononucleosis. However, little is known about the virologic and immunologic events that occur during the long incubation period prior to symptoms in adults or in asymptomatic individuals especially children. Further research is needed to determine means to predict who will be most severely affected, and to develop therapeutic strategies to reduce symptoms.

Secondly, the most severe consequences of EBV occur when the immune system fails to control infection and/or viral oncogenesis. The disease-causing role of EBV in XLP and PTLD patients is indisputable. Current knowledge of these diseases

has clearly positioned the field to undertake steps toward treatment, but there are few candidate antiviral drugs and much work remains to be done to make such therapies effective and practical. Increasing understanding of how specific EBV gene products and expression programs contribute to pathogenesis holds promise for more rational treatment strategies in the future.

Finally, an EBV vaccine could reduce the substantial disease burden due to primary EBV infection and possibly prevent or modify its chronic sequelae. However, development of an EBV vaccine has been agonizingly slow. More resources should be devoted to this endeavor, which has the potential to reduce the impact of a very common infectious disease and could even reduce incidence of certain human malignancies, such as Hodgkin's disease, endemic Burkitt's lymphoma, and nasopharyngeal carcinoma. While the latter questions are beyond the scope of this thesis, in the subsequent chapters, we sought to better understand symptomatic and asymptomatic primary EBV infection in humans.

**THESIS STATEMENT**

The introductory chapter 1 summarized current knowledge about EBV including gaps in knowledge. Furthermore, in 2010, at the conclusion of the 14th Biennial Conference of the International Association for Research on Epstein-Barr Virus and Associated Diseases, Dr Alan Rickinson gave a brief overview of the conference and identified future points of interest: 1) What happens in early primary infection? How is the switch to latency established? 2) What factors predispose to mononucleosis? 3) How does EBV get into T/NK (natural killer) cells? 4) What is the role of EBV in autoimmune disease? 5) Can we define a vaccine to prevent infection?

This thesis addresses aspects of the first two questions. First, what happens in early primary EBV infection (both symptomatic and asymptomatic)? Second, what are the immunologic (CD8 T cells, NK cells, cytokines) and virologic (viremia and virus in the oral cavity) correlates of disease severity; what is the association between CD8 lymphocytosis and NK cell expansion? Third, is the lymphocytosis seen during primary EBV infection all EBV-specific? Fourth and finally, is there attrition of pre-existing memory CD8 T cells to heterologous viral infections after primary EBV infection?

Thus, in Chapter 2, we set out to understand if a prospective study could be used to determine the immune responses that occur after primary EBV infection in asymptomatic adults. We next sought out to define the correlates of severe clinical illness and showed that viremia, CD8 T cell and NK cell expansion all correlated with disease severity. We highlight correlations with IL-6, an anti- and pro- inflammatory cytokine that may indicate a role for soluble factors created by the immune system in disease pathogenesis. In Chapter 3, we demonstrate that bystander T cells do become activated during primary EBV infection, but in general, do not expand. Finally, we show although the primary EBV response is quite robust in nature, attrition of pre-existing influenza or cytomegalovirus CD8 T cells did not occur.

# CHAPTER 2

## Insights from a prospective study of primary EBV infection: Viral load in the blood is not an irrelevant correlate of symptomatic infection

Figures and tables in this chapter subsequently used for publication in *Journal of Infectious Diseases*, Vol 207, Jan 2013, p. 80-88; Balfour HH Jr, Odumade OA, Schmeling DO, Mullan BD, Ed JA, Knight JA, Vezina HE, Thomas W, Hogquist KA. Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. doi: 10.1093/infdis/jis646. 

Primary infection with Epstein-Barr virus (EBV), a latent chronic herpes virus, may result in asymptomatic seroconversion or can lead to the development of clinical disease known as infectious mononucleosis. While there is a robust T cell response composed mainly of CD8+ lymphocytes, it is still unclear if lymphocytosis or virus is the primary driver of acute disease. To be able study both the symptomatic and asymptomatic EBV-specific immune response, we performed a longitudinal prospective study of EBV naïve individuals undergoing natural infection. In contrast to previous studies, these results indicate that infectious mononucleosis occurs in the majority of newly infected adults, with a broad range of severity. In addition to defining the kinetics of oral and peripheral blood viral replication, plasma cytokine responses, natural killer (NK), CD8+ T and CD4+ T cell activation and/or cell expansion, we identify potential correlates of severe disease. In contrast to previously published data that negate a role for EBV itself, our results indicate that viremia is associated with an expansion of CD8 and NK lymphocytes and correlates positively with disease severity. Thus, these data suggest an important role for viremia in clinical illness in support of developing antivirals for controlling EBV-induced infectious mononucleosis.

## Introduction

Epstein-Barr virus (EBV) is a double-stranded DNA gamma-herpesvirus that is one of the eight known human herpesviruses [121]. EBV is highly prevalent in the human population, and usually transmitted through virus-contaminated saliva. Like other herpesviruses, primary EBV infection results in acute infection followed by viral latency and lifelong infection [121]. Although there are no animal models of primary EBV infection, multiple clinical and retrospective studies have been performed in order to understand the human antiviral immune response to a chronic virus [121,178,179]. The complex interactions between the immune system and EBV result in a spectrum of disease presentations that can range from asymptomatic, to acute disease, to propagation of tumors [121,178,179].

The most common acute disease caused by primary infection with EBV is infectious mononucleosis (IM). Clinically, the predominant signs and symptoms include pharyngitis and sore throat, cervical lymphadenopathy, fatigue, headache, and fever [178]. Unlike many viruses that induce lymphopenia, the predominant attribute of infectious mononucleosis is elevated white blood cell counts, made up largely of atypical blasting CD8+ T cells. Currently, there is no treatment except supportive care, and sustained symptoms including fatigue result in loss of productivity for students, military personnel and adult professionals.

The incidence of IM in response to primary EBV infection is thought to increase with age [121]. In developing countries, where seroprevalence is often almost 100% in the first 2 years of life, EBV infection occurs without clinical symptoms. However, later infection around the second decade of life, as occurs in the United States and Europe, often leads to development of infectious mononucleosis in approximately 25% of cases [121]. The reason(s) for this dichotomy is largely unknown although there have been many speculations.

It has been postulated that IM is an immunopathologic disease, where symptoms are caused by the massive CD8 T cell response to the virus, and not the virus per se. Indeed, CD8 lymphocytosis, and not viremia (viral load in the blood) was associated with development of symptomatic disease in a previous study of primary EBV infection [130]. Furthermore, treatment of IM patients with antiviral drugs reduced viral load in some cases but generally did not alter the disease course [58,154-158,160]. An attractive model has been put forth, based on the concept of "heterologous immunity", in which memory T cells specific to other pathogens can cross-react with EBV and contribute to the lymphoproliferation that characterizes this disease [135]. In support of this model, T cells that cross-react with EBV-BMFL1 and Influenza-M1 were identified by stimulating T cells specific for either EBV or Influenza-M1 with peptides for the converse virus [135,180]. Based on these studies, the Welsh group created a matrix of CD8 T cell cross-reactivity that they suggest contributes to development of infectious mononucleosis [135,180]. This model proposes that repeated exposure to influenza (or other pathogens) during childhood results in the overzealous CD8 T cell response that characterizes IM in adults. Consequently, to date, infectious mononucleosis is considered to be an immunopathologic disease caused by the CD8 T cell adaptive immune response.

Here we show the findings of a prospective study of primary EBV infection, which we argue is a better study design (versus retrospective studies) for understanding infectious mononucleosis and the acute phase of the immune response to chronic virus infections in humans. In comparison to the clinical study model, we found there is a broad disease expression spectrum during primary EBV infection. We measured blood and oral viral loads, CD8, CD4, and NK cell numbers, as well as T cell activation markers (CD38, HLA-DR, and Granzyme B) to define the range and kinetics observed during primary infection in young adults and correlate them to disease severity. Surprisingly, the data showed a positive correlation between disease severity and blood

viremia, CD8 lymphocytosis, and NK cell expansion. Thus, the data support a revised model in which, viremia may result in cytopathic effects, activation of innate and adaptive immunity, and in an inflammatory response that results in development of clinical disease.

## Results

### Comparison of a clinical study versus a prospective analysis of EBV infection in young adults

For the prospective study, incoming college freshman at the University of Minnesota were screened for EBV-specific antibodies using a quantitative Enzyme-Linked ImmunoSorbent Assay (ELISA) assay. Those who had not been previously exposed to EBV were recruited to the study. Subsequently, enrolled study subjects were screened for exposure to EBV on an ongoing basis. Blood and/or oral throat wash samples were taken every 4-8 weeks and then evaluated for EBV genomes by qPCR and/or for antibodies to EBV viral antigens. Infection with cytomegalovirus and herpes-simplex virus were documented in some individual with either qPCR or via presence or absence of IgG antibodies for these viruses.

In order to characterize disease severity on a numerical scale, we utilized a previously published scale, [48] based on how much physical activity was limited (0-3) and the intensity of symptoms/pain (0-3) (Table 2-1). This resulted in a range from 0 (completely asymptomatic) to 6 (essentially bedridden) for disease severity. Symptoms were recorded using an online reporting system, as well as noted by nurses in the clinical record. Put together, we were able to determine the duration of illness and calculate a clinical score (Table 2-2) based on the top rated signs and symptoms attributable to EBV infection [178].

| Table 2-1. Severity of Illness scale* | |
|---|---|
| Category | Point(s) |
| *Physical activity limitation* | |
| No limitation | 0 |
| Attends all classes but fatigues easily | 1 |
| Misses some classes | 2 |
| Cannot go to class | 3 |
| *Symptom/pain intensity* | |
| None | 0 |
| Sometimes bothersome but does not limit activities | 1 |
| Bothersome all the time when awake and limits some activities | 2 |
| Severe enough to interfere with sleep and limits most activities | 3 |
| **Total possible score** | **6** |
| **Reprinted from the *Journal of Infectious Diseases.* 2005 September 26; 192: 1505-1512. Balfour et al. "A prospective clinical study of Epstein-Barr virus and host interactions during acute infectious mononucleosis". [48] | |

**Table 2-1: Severity of illness during primary EBV infection was evaluated using a previously established 2-prong criterion.** Both physical activity limitation and symptom/pain intensity are evaluated to create the severity of illness score. Scores range from 0 (completely asymptomatic) to 6 (essentially bedridden)

| Table 2-2. Clinical Score | |
|---|---|
| **Sign or Symptom** | **Weight Given:** |
| Sore throat | 2 |
| Lymphadenopathy | 2 |
| Fatigue | 2 |
| Headache | 1 |
| Fever | 1 |
| Decreased appetite | 1 |
| **Total possible Clinical Score** | **9** |

**Table 2-2: A weighted composite of six signs or symptoms was used to calculate clinical score during primary EBV infection.** Scores range from 0 (asymptomatic) to 9.

Of 147 subjects followed in the prospective study for 4-5 years, 66 experienced primary EBV infection. The sample collection time-points for those 66 are shown in Fig. 2-1 with samples collected during the 42 days prior to symptom onset in 27 subjects, which is the putative incubation period of the virus [181].

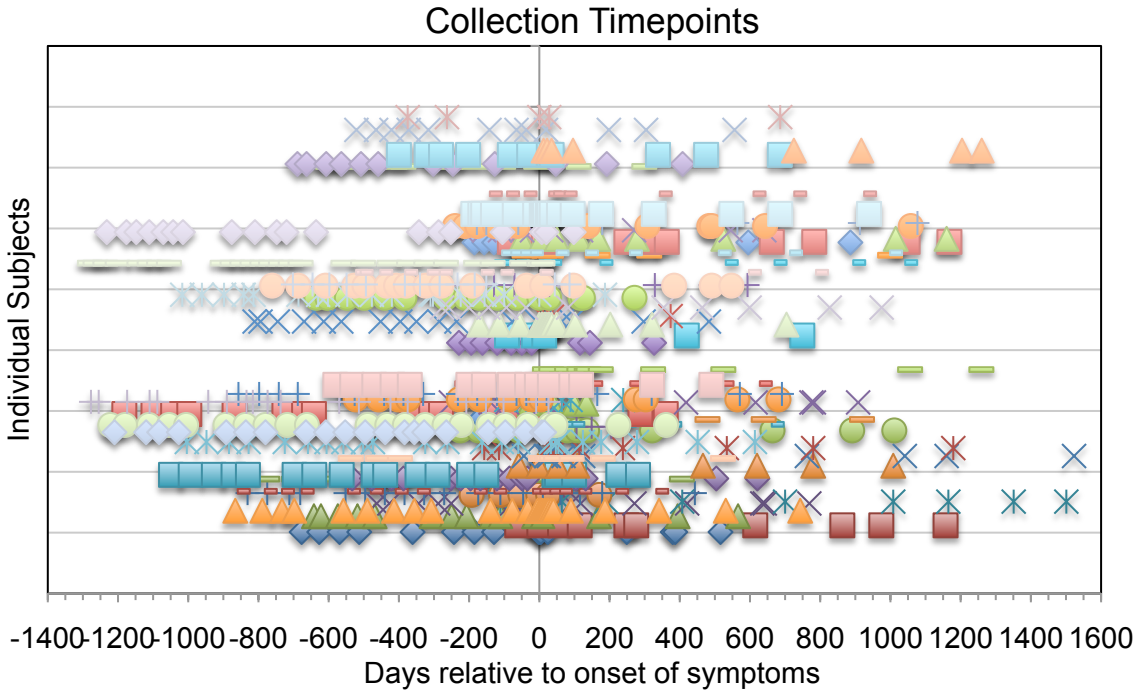


**Figure 2-1. Prospective analysis allowed sample collection before, during, and after primary EBV infection in young adults**. Blood samples were obtained from each subject at prior to, during and after primary EBV infection (n=67). For each subject, there was a minimum of 3 and a maximum of 28 collection timepoints.

We compared disease severity in subjects in the prospective study with that of subjects who were in the control groups of previous clinical studies referred by the University of Minnesota Boynton Health services clinic with a clinical diagnosis of primary infectious mononucleosis [58]. We observed lower severity of illness scores (prospective median=3, clinical median=5, $p<0.001$) and shorter disease duration for subjects in the prospective study compared to those who sought health services in response to primary EBV infection (Fig. 2-2). In conclusion, clinical studies presented a

skewed perspective for understanding acute EBV infection in that they underestimate the incidence of IM and are composed of the sickest of subjects.

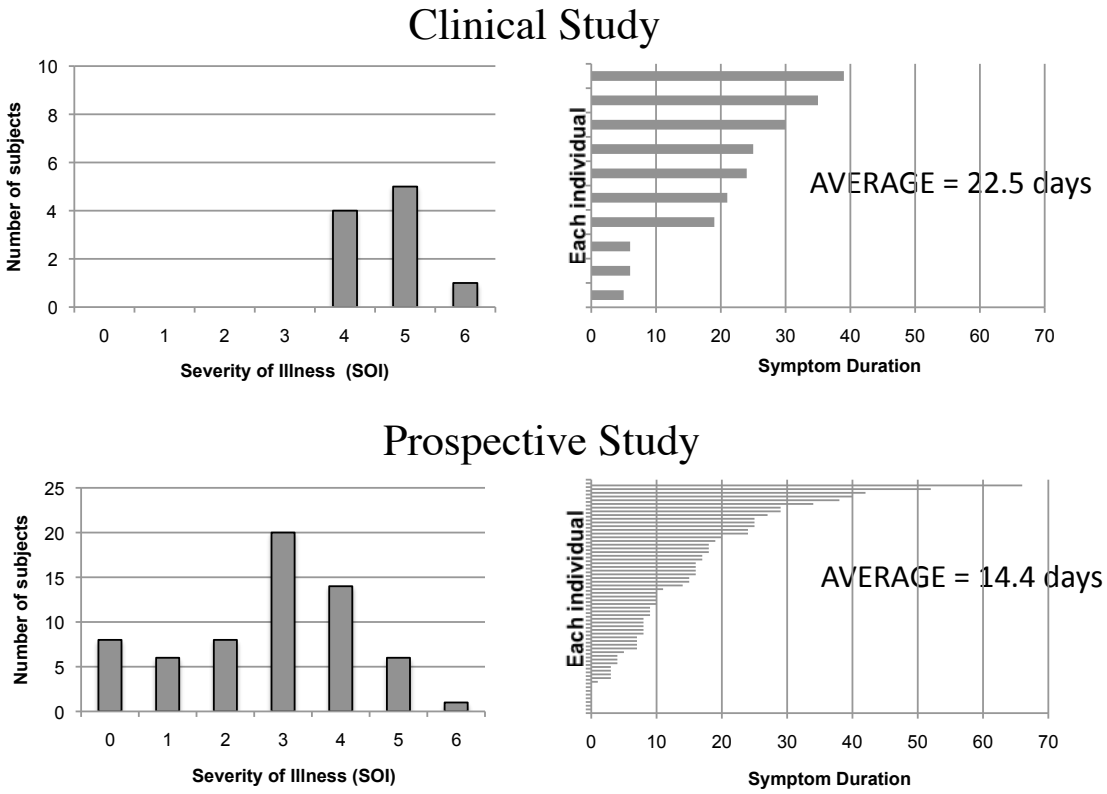


**Figure 2-2: There is a broader range and a shorter duration of disease severity in a prospective primary EBV study in contrast to a clinical study**. Graphs represent the number of individuals with each severity of illness score (left) and the duration of illness (right) for each individual (top) in a clinical study and (bottom) in a prospective study. (n=10; n=62 respectively). $p<0.001$ for symptoms duration between the clinical and the prospective study.

**Identifying the kinetics of T cell activation during primary EBV infection**

Multiple studies have previously shown that CD8 T cell numbers in the peripheral blood increase dramatically during acute primary EBV infection [121], often termed "CD8 lymphocytosis". Since lymphocytosis has been previously suggested to be an important factor in disease severity [130], we quantified CD8 T cell numbers over time in prospective study subjects. Because cell numbers recovered from blood were variable between individuals, we found the data to be more consistent when graphing the ratio of CD8 T cells to CD4 T cells in each patient sample. As shown in Fig 2-3, there was no significant difference between using the ratio of CD8 to CD4 T cells and the total number of CD8 T cells, reflecting the fact that CD4 T cell numbers did not increase during acute IM [182].

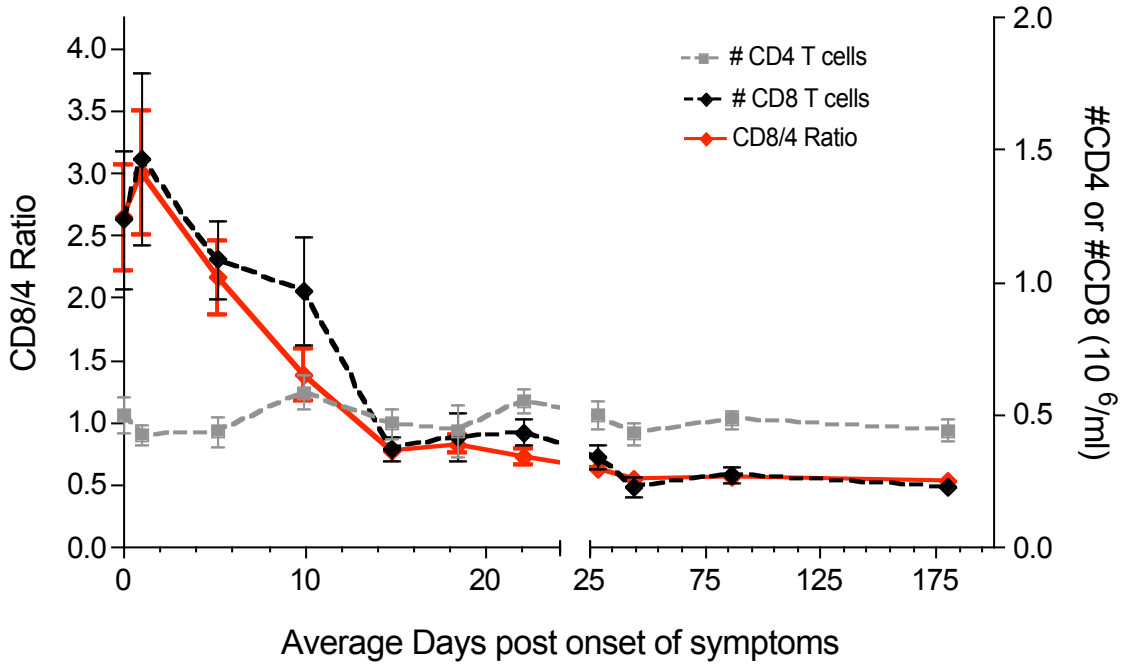


**Figure 2-3: The ratio of CD8 T cells to CD4 T cells is an appropriate surrogate for total CD8 T cell counts.** PBMCs from each subject were stained with antibodies against CD3, CD4, and CD8. To calculate total cell numbers, the frequency of CD3+ CD8 T cells and CD3+ CD4 T cells relative to the live gate was calculated using FlowJo (Treestar) and then multiplied by the PBMC count per milliliter of blood. Gated frequency of T cells in the CD3+ gate was used to calculate the ratio of CD8 T cells to CD4 T cells. Error bars represent standard error of the mean (n=22).

Fig. 2-4A shows data from various time points of subject #5036 as an example, where the CD8/4 ratio is 0.23 prior to infection, increases to 8.1 during IM, and resolves to 0.32 after 40 days.

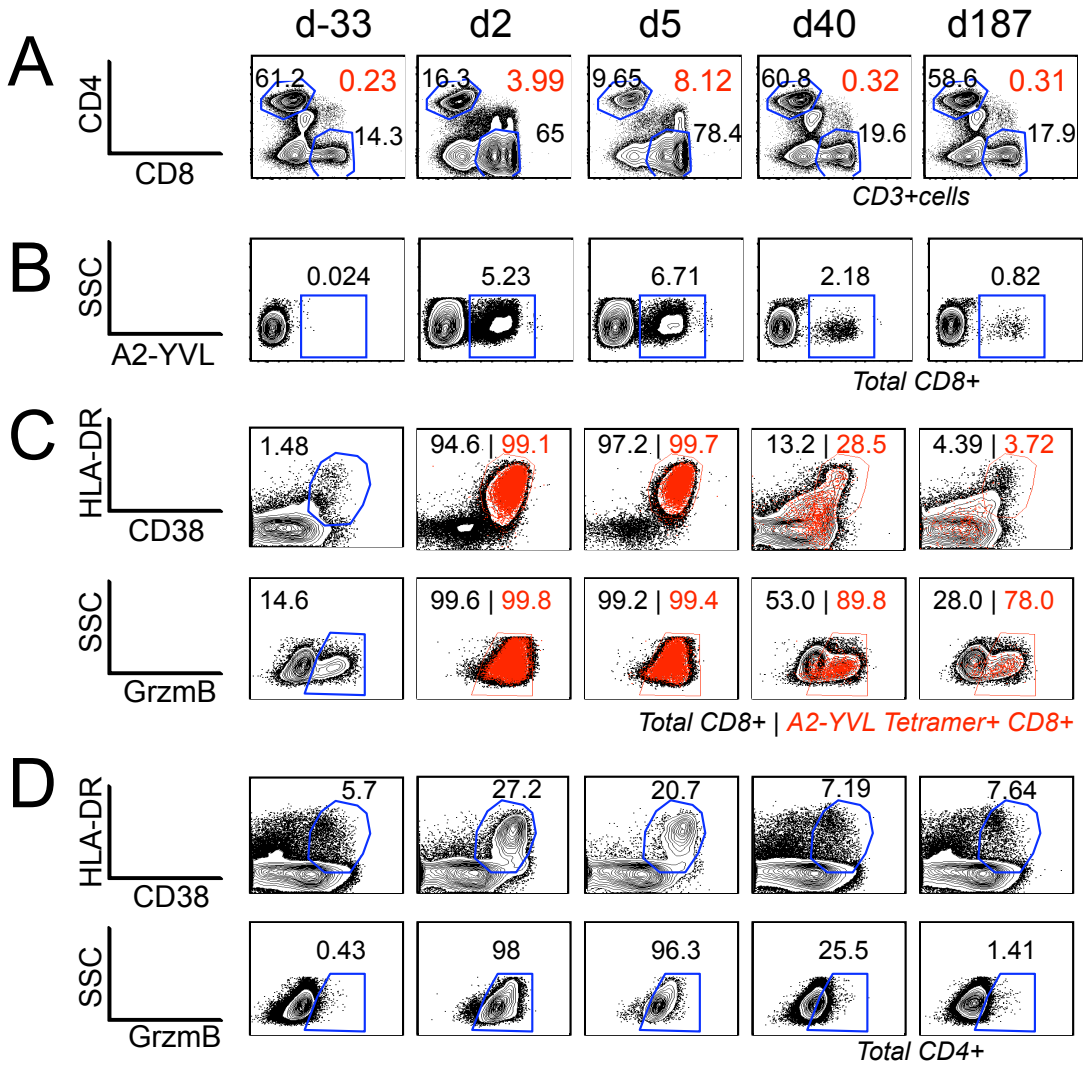


**Figure 2-4: EBV specific T cells expand and contract, however, a majority of total CD8 T cells showed a classic expansion/activation pattern during acute infection (while a smaller percentage of CD4 T cells are activated).** Sample multi-parameter flow cytometric analysis of PBMCs from one subject prior to, during and after acute infection with EBV. (A) CD4 and CD8 expression on CD3 T cells over time. (B) CD3+ CD8 T cells specific for the HLA-A2/YVL epitope identified using peptide-MHCI tetramers. (C) Dot plots showing activation markers, CD38 and DR (top) and granzyme B (bottom) expression over time on total CD3+ CD8 T cells (black) and A2-YVL tetramer+ CD3+ CD8 T cells (red). (D) Dot plots showing activation markers, CD38 and DR (top) and granzyme B (bottom) expression over time on total CD3+ CD4 T cells.

We used EBV human leukocyte antigen (HLA)-restricted class I tetramers to determine the evolution of the EBV-specific CD8 T cell response (Fig. 2-4B). Furthermore, we performed phenotypic analysis of both total and EBV specific CD8 and CD4 T cells using activation markers HLA-DR (DR) and CD38, and a marker of cytolytic activity, granzyme B (GrzmB). These markers have been used to characterize the magnitude and kinetics of effector phenotype T cells for the yellow fever virus (YFV)-17D and the smallpox (Dryvax) vaccine studies [183] and T cell activation in other viral infections [184,185]. In this cohort, we observed a significant upregulation CD38 and DR on CD8 T cells, and to a lesser extent on CD4 T cells. This occurred primarily within the first 4 weeks of symptom onset (Fig. 2-4C and Fig. 2-5).

Unlike CD38 and DR, which are expressed at lower levels prior to infection in CD8 T cells (average=3.65% $\pm$ 4.35%), we observed a higher baseline expression of GrzmB in CD8 T cells (average=14.48% $\pm$ 9.83%) ($p<0.001$). However, there was a significant upregulation of GrzmB on both CD8 T cells (average-64% $\pm$ 30%) and CD4 T cells (average-33% $\pm$ 32%) during acute disease ($p<0.001$) and to our surprise, in some individuals, CD4 T cells seemed to upregulate GrzmB levels to the same degree as CD8 T cells (Fig. 2-4D and Fig 2-5). Finally, downregulation of GrzmB on CD8 T cells occurred at a slower rate relative to that of DR and CD38 co-expression (Fig. 2-5). That the kinetics of phenotypic changes as CD8 T cells transition from the effector phase to the memory phase varied for individual markers is not surprising [183], however, it was interesting that CD4 T cell downregulation of GrzmB occurred at a similar rate as the reduction of CD38/DR on CD4 and CD8 T cells.

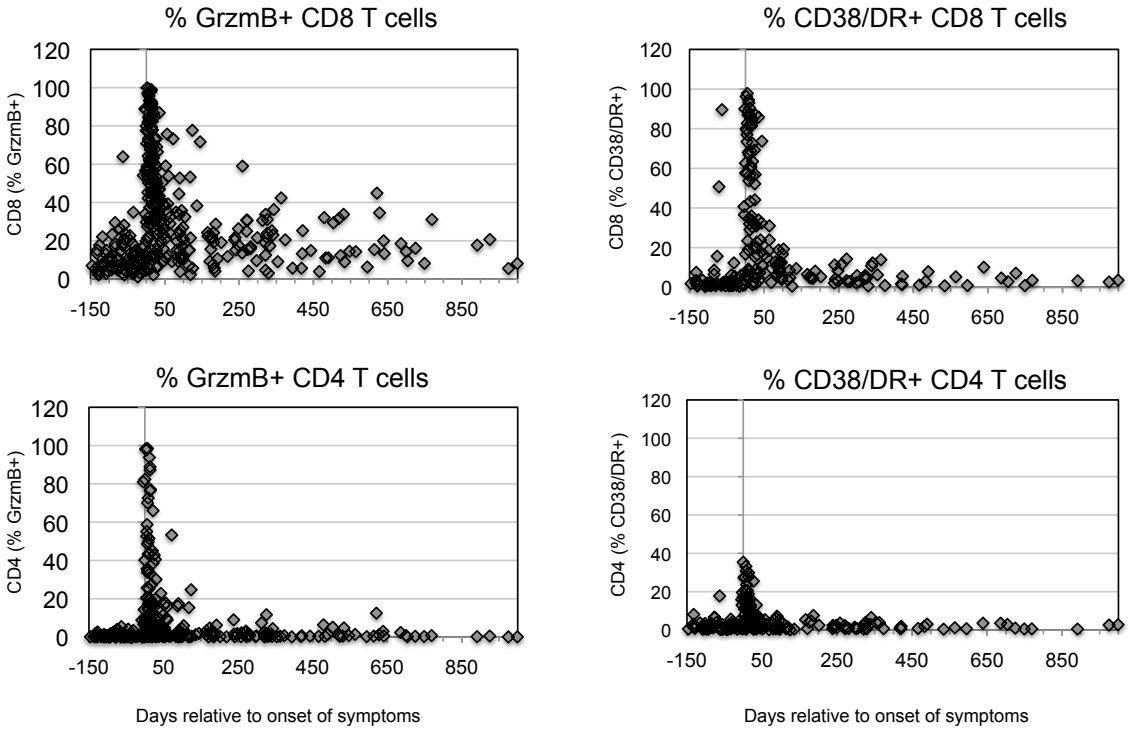


**Figure 2-5: In contrast to CD4 expression of GrzmB or CD38/DR, CD8 T cells express elevated GrzmB levels, but not CD38/DR after acute primary EBV infection.** PBMCs from each subject prior to, during and after acute infection with EBV were stained with antibodies to CD3, CD4, CD8, HLA-DR, CD38 and granzyme B. (Top) Granzyme B expression (left) and activation markers, CD38 and DR co-expression (right) over time on total CD3+ CD8 T cells in all subjects. (Bottom) Granzyme B expression (left) and activation markers, CD38 and DR co-expression (right) over time on total CD3+ CD4 T cells in all subjects. (n=40-58)

**Identifying the kinetic window for lymphocytosis, and viral load in both the oral cavity and peripheral blood during primary EBV infection**

Overproliferaton of CD8 T cells causing an immunopathologic response versus viral load in the blood, resulting in viral cytopathy have been suggested by previous studies as the potential basis of infectious mononucleosis. Therefore, we investigated the correlations between lymphocytosis, viremia and disease severity. To do this, we first defined the most appropriate kinetic window in which to evaluate lymphocytosis and viremia during acute primary EBV infection (Fig. 2-6). The CD8 T cell to CD4 T cell ratio

(CD8/4) of the entire cohort, which was ~0.5 prior to infection, rose to an average of 3-fold in the first 30 days after symptom onset.

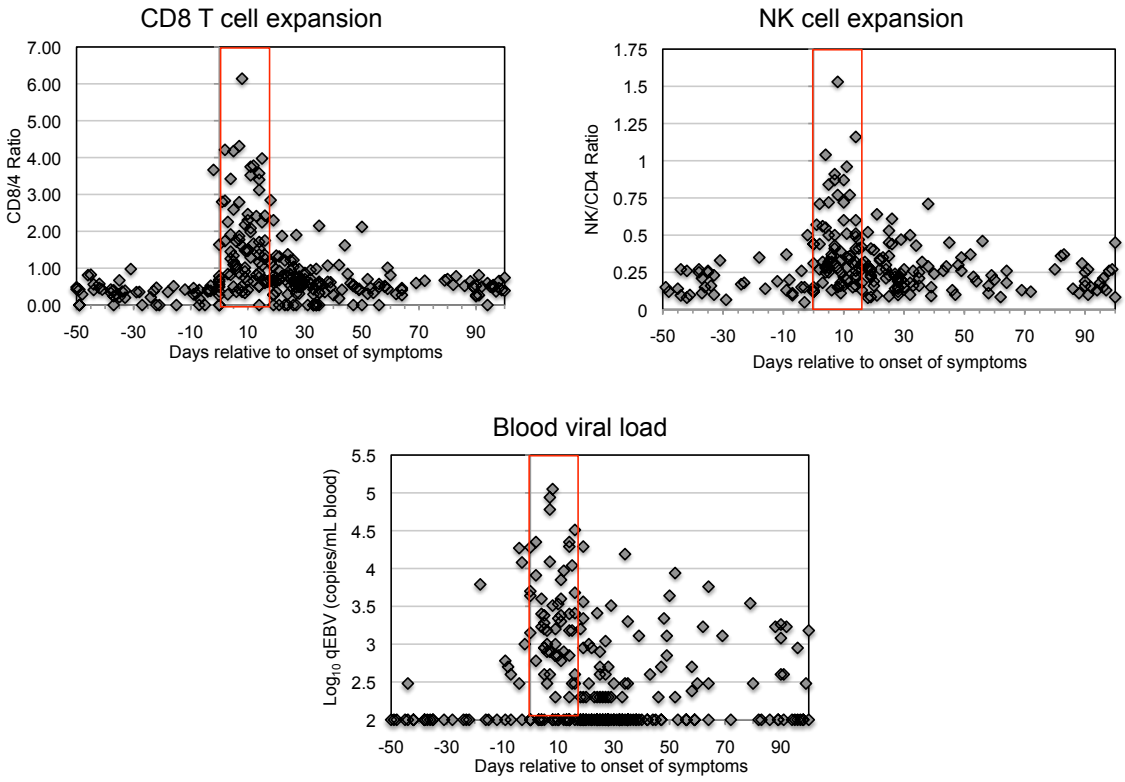


**Figure 2-6: Lymphocytosis (CD8 T cell and NK cell) and viremia occur over a limited time frame during acute infection.** (Top) PBMCs from each subject prior to, during and after acute infection with EBV were stained with antibodies to CD3, CD4, CD8, and CD56. (top left) CD8/CD4 ratio and (top right) CD56/CD4 ratio over time on total CD3+ T cells and CD3- cells respectively, in all subjects. (Bottom left) EBV blood viral load copies/ml quantitated by PCR in all subjects. Time window depicted here is from 50 days prior up until 100 days after onset of symptoms. Red boxes highlight peak of parameter indicated. (n=40-58)

Although many studies have determined the incubation phase of EBV to be ~40 days [181], we rarely detected viremia or virus in oral fluids prior to the onset of symptoms (Fig. 2-7). For most individuals, EBV was no longer detectable after 200 days in the blood; however, we observed sustained virus in the oral cavity on a continuous basis.

Moreover, since oral EBV shedding in the same individual can vary by 4 or 5 logs [17], not everyone had detectable oral virus during each collection time point.

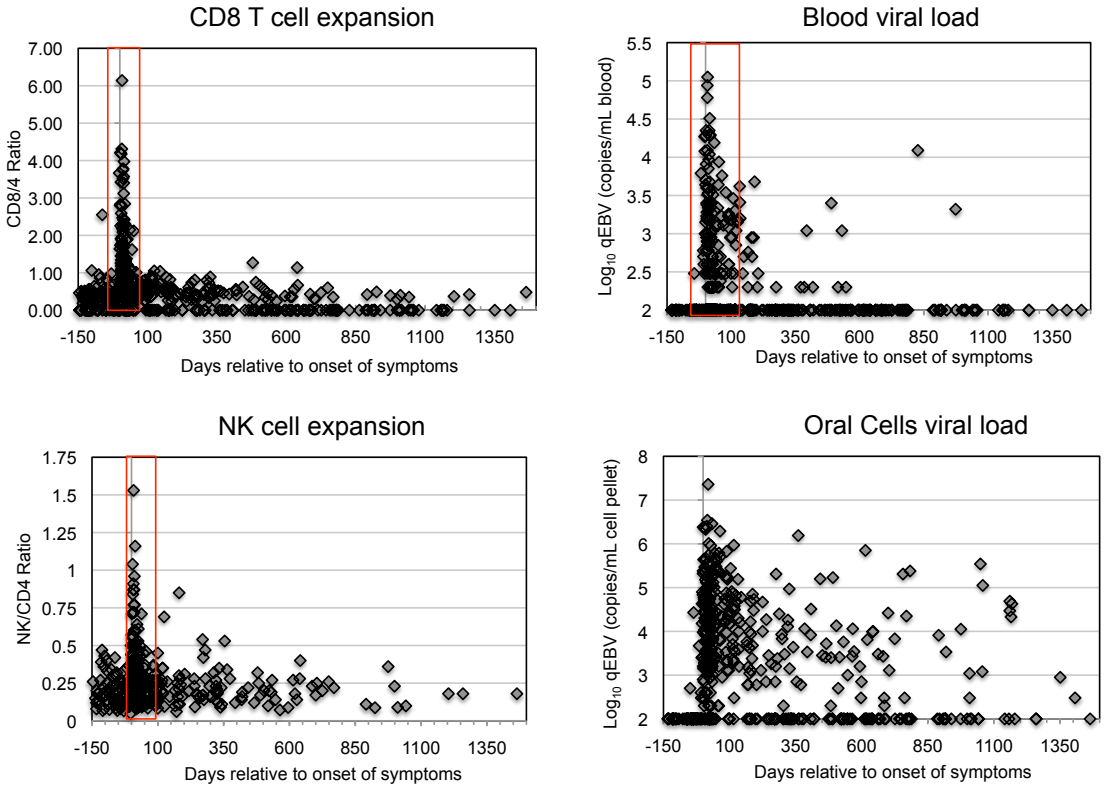


**Figure 2-7: The longitudinal prospective study shows limited viral shedding into the blood and limited changes in CD8 lymphocytosis and NK cells expansion after acute primary EBV infection is resolved.** (Left) PBMCs from each subject prior to, during and after acute infection with EBV were stained with antibodies to CD3, CD4, CD8, and CD56. (top) CD8/CD4 ratio and (bottom) CD56/CD4 ratio over time on total CD3+ T cells and CD3- cells respectively, in all subjects. (Right) EBV viral load copies quantitated by PCR (top) per ml of blood and (bottom) per ml of oral cell pellet in all subjects. Time window depicted here is from 150 days prior up until 1500 days after onset of symptoms. (n=40-58)

Finally, in order to perform a comprehensive study to determine correlates of disease severity, we also determined the expansion and activation of NK cells during acute primary EBV infection. NK cells have been previously shown to expand during acute infectious mononucleosis [182]. In a previous smaller study by Williams et al., (n=3

per group), individuals who experienced a higher NK cell elevation from their post-IM baseline (80 days) had more severe sore throats [106]. In this study, NK cell elevation is expressed as the ratio of NK cells to CD4 T cells (NK/CD4), a value that reflects the total numbers of NK cells since the number of CD4 T cells does not change during primary EBV infection (Fig. 2-3). While the activation of NK cells, as determined by GrzmB, was not significantly different before and during acute primary EBV infection (data not shown), we observed an expansion of NK cells during acute disease (Fig. 2-6 and Fig. 2-7). Furthermore, we identified a window of NK cell expansion (within 30 days after symptom onset) that was identical to that observed for CD8 lymphocytosis (Fig. 2-6).

**Viral load in the blood is not an irrelevant factor for disease severity during IM**

Since lymphocytosis is characteristic of infectious mononucleosis, we first determined if it correlated with severity of illness (SOI) during the first 15 days after symptom onset. We observed a strong positive association between severity of illness and the CD8/4 T cell ratio (Spearman $r$ =0.59), CD8 T cell CD38/DR expression (Spearman $r$ =0.73) and CD8 T cell GrzmB expression (Spearman $r$ =0.37) (Fig. 2-8). Next, since we had previously determined that CD4 T cells also upregulated CD38/DR and GrzmB during acute primary EBV infection and since it has been suggested that CD4 T cells specific for lytic antigens may help control virus replication [121], we tested for correlations between SOI with the frequency of CD4 T cells expressing GrzmB or co-expressing CD38/DR. Interestingly, while CD4 CD38/DR positive cells correlated positively with SOI (data not shown, Spearman $r$ =0.67), CD4 GrzmB expression did not (Fig. 2-8). Therefore, consistent with previously published data, we found a strong positive correlation between CD8 lymphocytosis and disease severity.

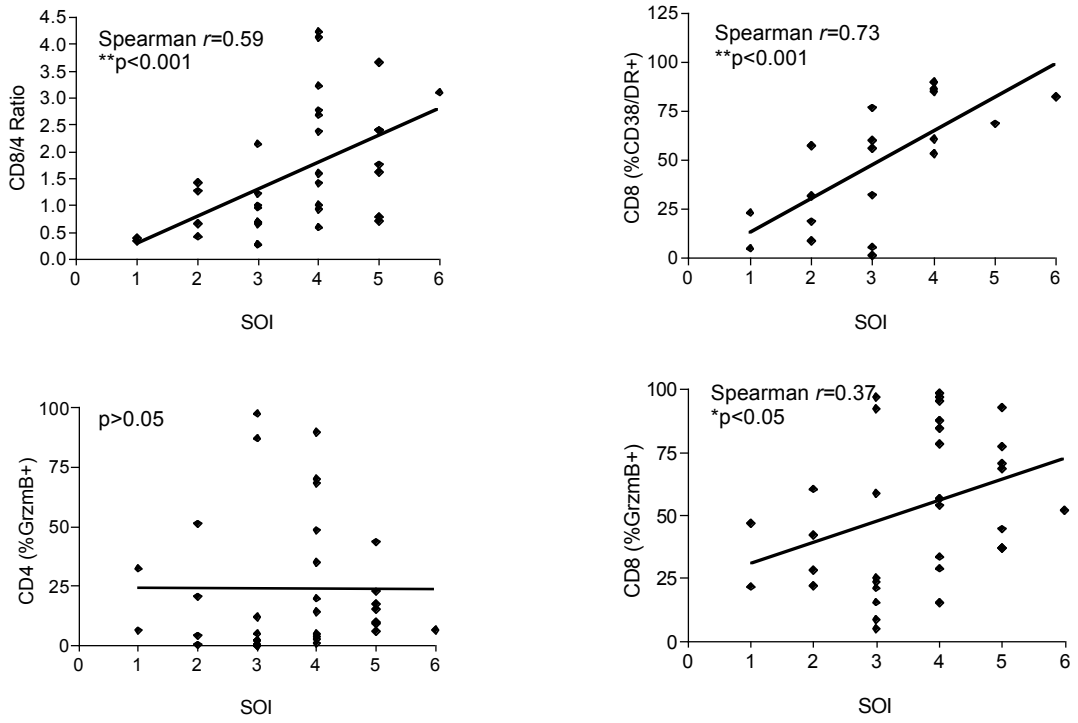


**Figure 2-8: There is a strong correlation between disease severity and CD8 lymphocytosis and CD8 activation.** PBMCs from each subject prior to, during and after acute infection with EBV were stained with antibodies to CD3, CD4, CD8, HLA-DR, CD38 and granzyme B. Graph shows the correlation between CD8/CD4 ratio (top left, n=34), CD38/DR+ CD8 expression (top right, n=20), GrzmB+ CD4 expression (bottom left, n=34), GrzmB+ CD8 expression (bottom right, n=34) and severity of illness (SOI).

Subsequently, we wanted to determine if viral load would be associated with disease severity. Contrary to a smaller clinical study [130], we observed a positive correlation (Spearman *r* =0.41) between viremia and severity of illness (Fig. 2-9A). Although EBV is transmitted through the oral cavity, we did not observe a positive (or negative) correlation between oral viral load and SOI (Fig. 2-9A). If most or all of the CD8 T cell expansion and activation is EBV-specific, as has been suggested by others [121 186], it is possible that CD8 lymphocytosis is driven by the high viral load. In agreement with this, we detected a strong positive correlation (Spearman *r* =0.47) between blood (but not oral) viral load and CD8 lymphocytosis (Fig. 2-9A). Similar correlations were observed between clinical score, which is based on signs and

symptoms, and lymphocytosis (Spearman *r* =0.63), viremia (Spearman *r* =0.35) but again not with oral viral load (Fig. 2-10).

Finally, consistent with a previous study suggesting NK cell expansion was associated with more severe disease, NK/CD4 ratio correlated positively with SOI (Spearman *r* =0.42) (Fig. 2-9B). Furthermore, the NK/CD4 ratio also correlated positively with blood (Spearman *r* =0.45) but not oral viral load (data not shown). For each individual, the maximum NK cell expansion occurred in those with the maximum CD8 T cell expansion (Fig. 2-9B) suggesting that the same singular mechanism is responsible for driving expansion of both populations to create the immunopathologic response characteristic of infectious mononucleosis.

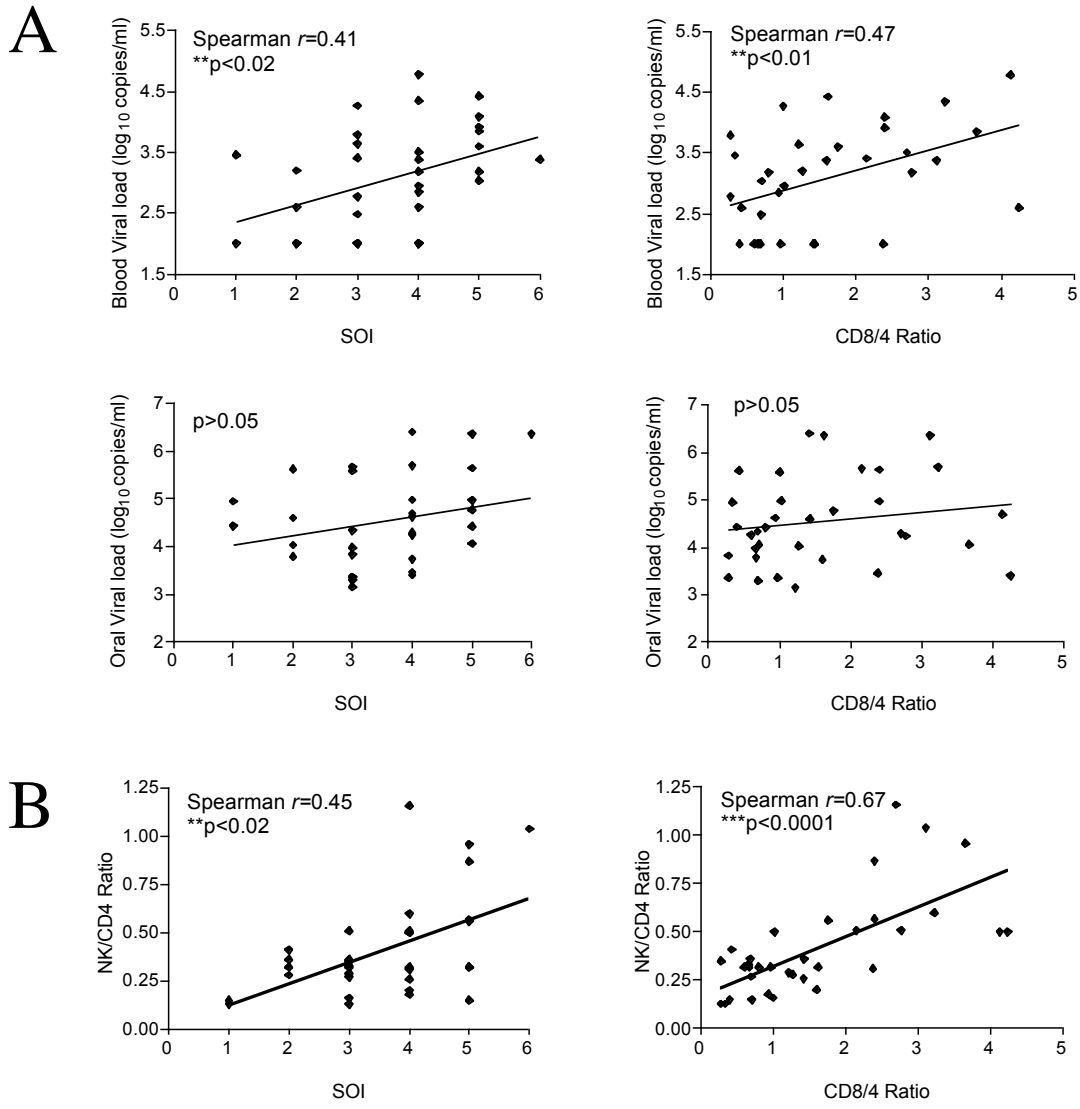


**Figure 2-9. The strong correlations between SOI and viremia and NK expansion suggest that viremia is a primary driver of IM**. (A) EBV viral load copies quantitated by PCR (top) per ml of blood and (bottom) per ml of oral cell pellet in all subjects. Left graphs show the correlation between blood viral load (top left) or oral viral load (bottom left) and severity of illness (SOI). Right graphs show the correlation between blood viral load (top right) or oral viral load (bottom right) and CD8 lymphocytosis. (B) PBMCs from each subject prior to, during and after acute infection with EBV were stained with antibodies to CD3, CD4, CD8, and CD56. Graphs show the correlation between NK cell expansion and severity of illness (SOI) (left) or CD8 lymphocytosis (right). (n=34) * p<0.05, **p<0.02, ***p<0.0001.

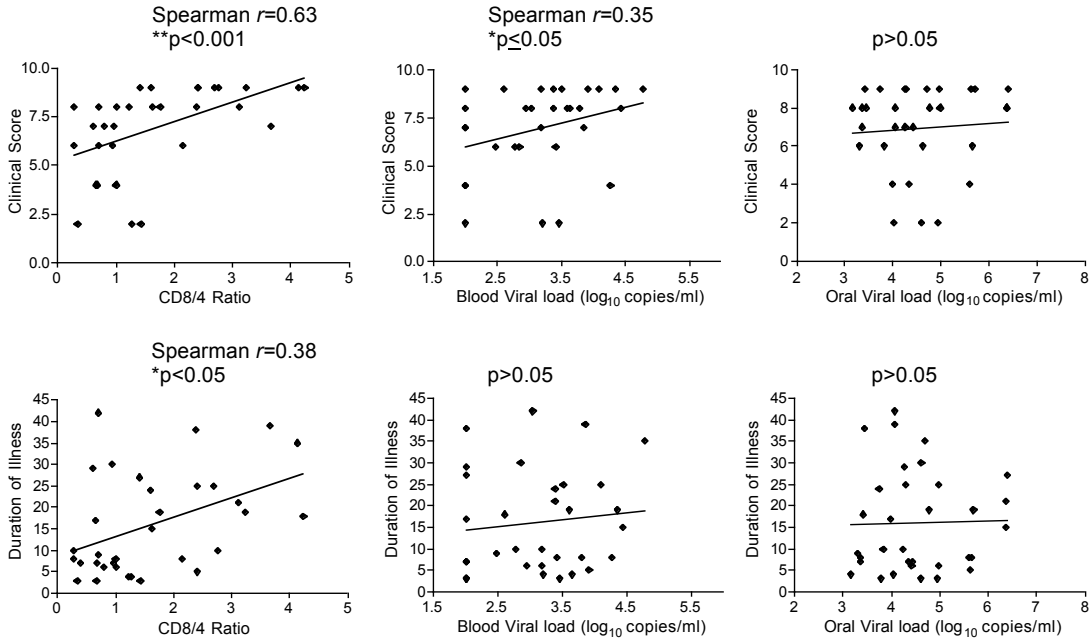


**Figure 2-10: Lymphocytosis is associated with disease severity, clinical score, and duration of illness while viremia strongly correlates with disease severity and clinical score**. (Left) Graphs show the correlation between CD8 lymphocytosis and clinical score (top) or duration of illness (bottom). (Middle) Graphs show the correlation between viral load copies per ml of blood and clinical score (top) or duration of illness (bottom). (Right) Graphs show the correlation between viral load copies per ml of cell pellet and clinical score (top) or duration of illness (bottom). (n=34) *p<0.05, **p<0.02, ***p<0.0001.

### IL-6, and not IFN-γ, correlates with disease severity

In addition to having cytotoxic potential, EBV-specific T cells make IFNγ during acute EBV infection [121]. Indeed, IFNγ can cause symptoms similar to that observed during infectious mononucleosis [187,188]. Therefore, we hypothesized that IFN may correlate with disease severity. However since several cytokines are elevated during IM, we identified 8 cytokines that might contribute to disease pathogenesis (discussed in Chapter 1). The cytokines studied were composed of antiviral cytokines (type I and II interferons (IFNs)), inflammatory/activating cytokines (interleukin-6 (IL-6), IL-12, tumor necrosis factor (TNF)-α) and suppressive cytokines (IL-10, transforming growth factor (TGF)-β).

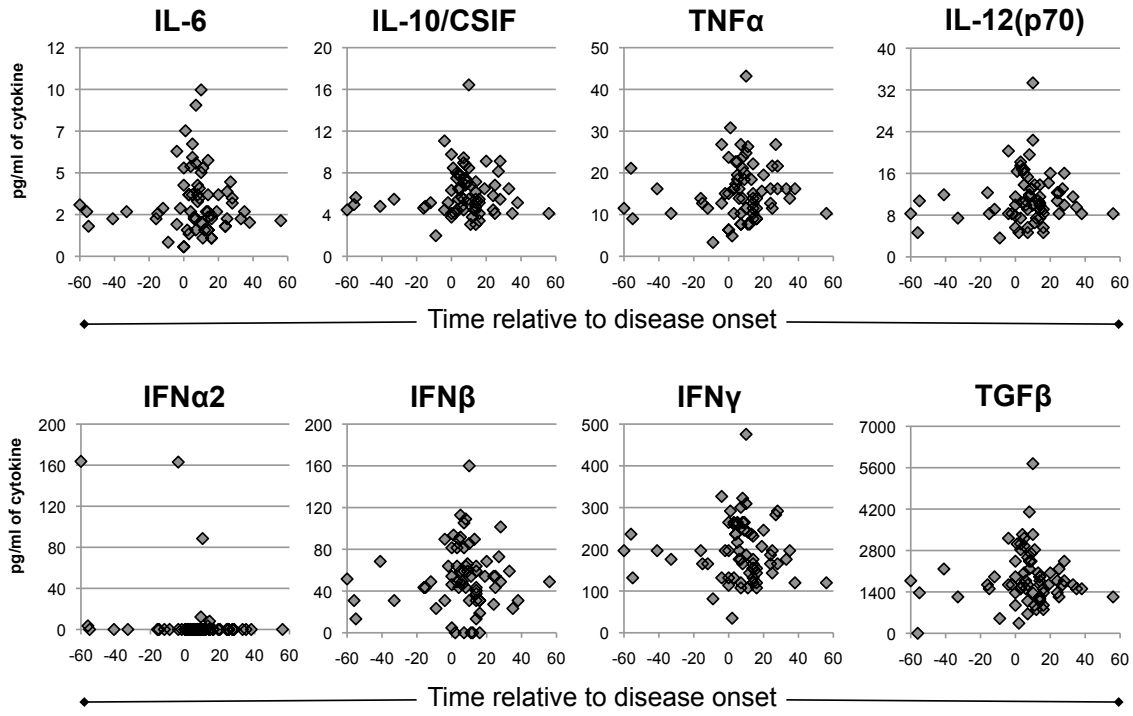


**Figure 2-11: Multiple plasma cytokine levels were found to be elevated during primary EBV infection**. The levels of 8 cytokines were measured in plasma using a luminex-based assay. The graphs here represent in pg/ml of cytokine in a time window from 60 days prior up until 60 days after onset of symptoms. (n=42)

While several cytokines were elevated during acute infection (Fig. 2-11), cytokine levels did not correlate significantly with CD8 lymphocytosis (data not shown). In individuals whose maximum viremia was greater than 3.5 $\log_{10}$ copies/mL, IL-6 levels were significantly higher (63% increase, 4.43pg/ml, p=0.008) and so were IL-10 levels (42% increase, 7.86pg/ml, p=0.002) versus individuals with viremia less than 3.5 $\log_{10}$ copies/mL (IL-6 = 2.72pg/ml; IL-10 = 5.52pg/ml). Furthermore, only interleukin-6 (IL-6) significantly varied in terms of severity of illness (Fig. 2-12), and clinical score (p=0.02, data not shown). For those with more severe disease (SOI score of 4-6), there was a significant elevation of IL-6 levels during IM (p<0.01). Interestingly, IFNγ levels did not correlate significantly with viremia, SOI, or clinical score (Fig. 2-12 and data not shown).

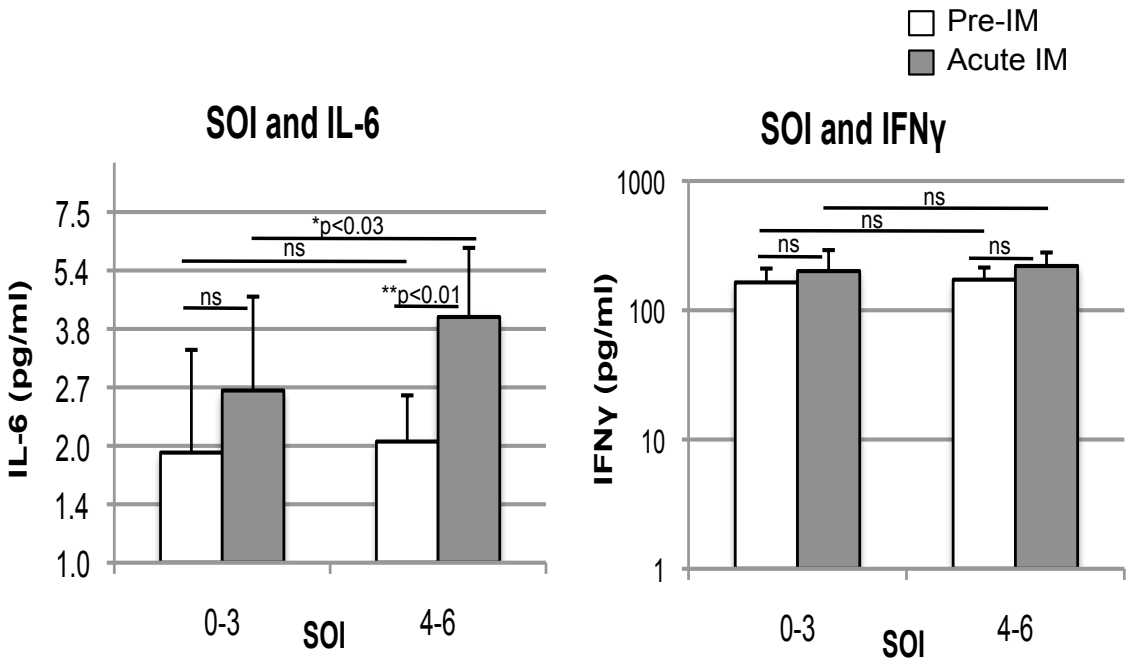


**Figure 2-12. Plasma levels of the innate cytokine IL-6 show a stronger correlation with disease severity than IFNγ.** The level of IL-6 and IFNγ were measured in plasma using a luminex-based assay. Subjects were divided into two groups based on severity of illness: mild or no disease (0-3) and severe disease (4-6). Pre-IM and acute IM values were calculated for each group. Unpaired student *t*-test was used to determine significance.

## Discussion

Despite the fact that the herpes family of viruses represent more than a quarter of chronic viral infections in humans [179], it is still unclear how to prevent infection and establishment of viral latency. Of relevance, EBV has been the chosen model for understanding the immune response to a persistent chronic virus [121]. The field's understanding of the immune response to primary EBV infection is derived from studies that enroll people who are often currently undergoing seroconversion and has, therefore, resulted in several questionable conclusions. Given the limitations of retrospective studies, we performed a prospective analysis of adolescent college students undergoing primary EBV infection due to natural exposure. The findings of this prospective analysis have modified the picture of IM to suggest that viral load in the blood is a critical determinant, and therefore a valuable target for therapeutics aimed at prevention or treatment of EBV-related pathogenesis.

Contrary to the data published in the field, we observed a greater incidence of IM in response to primary EBV infection in adults. Not surprisingly, IM is less severe than clinical studies suggest. Moreover, instead of a bimodal distribution of clinical symptoms, in young adults, infectious mononucleosis occurs in a uniform distribution with approximately only 10% of individuals experiencing asymptomatic seroconversion. In light of this finding, the notion that IM rarely occurs in children [121] may be a misconception due to lack of appropriately designed prospective studies and under-diagnosis. The increased duration of illness observed with a clinical study (compared to a prospective study) is likely due to the fact that those who seek relief from health care services have already had prolonged symptoms. Therefore, symptoms such as extended fatigue and loss of productivity associated with IM in adults may be more difficult to diagnose in children due to lack of recognition of prolonged disease. A serologic study of eighty infants (age 3-24 months) indicated that up to 50% are

seronegative for EBV [189]; however, there is a paucity of epidemiological studies that have investigated precise seroconversion rates with individuals in the first decade of life and beyond. In other countries, like China and Africa, EBV exposure occurred earlier with approximately 100% seroconversion of children (age <10 years). However, in 14 Chinese children with IM [190], a classic triad of fever, lymphadenopathy and tonsillar-pharyngitis, similar to adult IM [121] was observed. Since children are generally the optimal target age for immunization, it will be of great interest if future studies identify whether a similar range or duration of symptoms would be observed in children of different geographic locations, age and even socioeconomic status in the United States.

The findings of this prospective analysis also offer a clear kinetic picture of IM. Relative to most viruses, EBV has one of the longest incubation periods of viral infection described (~40 days). Therefore, this methodology allowed us to improve on historic studies[121] that have only been able to investigate the dynamics of the response from onset of symptoms to convalescence and the subsequent healthy carrier phase. We used the number of days post-onset of symptom(s) to normalize the longitudinal time-course data set. Surprisingly, increases in CD8 T cells number/frequency and blood viral load were generally not observed during the long (presumed) incubation period, but rather peaked close to the time of symptom onset (IM). This suggests that systemic infection/response rather than local (tonsillar) infection/response drive symptoms.

A recent study of the effector CD8 T cell response to smallpox and yellow fever vaccines suggest rapid optimal responses may preclude persistence of viruses and dictate whether an infection will be acute or chronic [183], although the definition of "optimal" may differ based on the pathogen (i.e. activation of naïve cells, suppression of effectors). Furthermore, elevated levels of programmed death-1 (PD-1), a marker often associated with T cell dysfunction,[191] were detected on total and EBV-specific T cells during acute infectious mononucleosis and was positively associated with a higher viral

load in B cells [192]. Therefore, during the early phase of infection, CD8 T cells may not be functionally equipped to control acute EBV infection. We find PD-1 induced-suppression unlikely because in one study, proliferation of EBV-specific CD8 T cells in response to peptide with and without blockade of the PD-1/PD-L1 interaction was minimal [192]. However, there are many other inhibitory receptors, such as CD244 (2B4) which co-regulate T cell exhaustion[191] and are also upregulated during acute infectious mononucleosis [182]. In line with our developing model, it is possible that the longer duration of virus in the blood may imply sub-optimal T cell efficacy during acute infection. Nonetheless, the data bring to light the potential role of viremia on development of IM.

Despite an inclusive role for adaptive immunity, the potential contributions of innate immune responses in EBV pathogenesis have yet to be clearly defined [193]. A key arm for early antiviral responses, natural killer (NK) cells are elevated during acute IM [182] and in a small study of 6 individuals, NK cell expansion was associated with severe primary EBV infection [106]. A more recent study suggests that early viral activation of dendritic cells results in production of IL-12, NK cell activation (especially tonsillar NK cells) and subsequent limitation of EBV-induced B cell transformation via IFN$\gamma$ [194]. Even though we were unable to investigate the role of tonsillar NK cells, we did detect an increase in blood NK cells numbers that was synchronous with the increase in CD8 T cell numbers. While the data does not preclude a model in which failure of tonsillar NK cells results in EBV propagation in peripheral blood, it would suggest a common factor driving both CD8 T cell and NK cell expansion. This is in contrast to a model that negates the impact of viral load and attributes the development of infectious mononucleosis to the CD8 T cell response alone [130].

To gain further insight into the pathogenesis of acute infection, we looked at correlates of disease severity. Consistent with the previous study [130], we observed a positive correlation with CD8 T cell lymphocytosis and severity of illness. Additionally,

based on the average duration of illness (14.4 days) and the general time frame within which CD8 lymphocytosis occurs (~16 days), the data did not initially refute the circumstantial conclusion that IM is an "immunopathogenic" disease (caused by the immune response to virus, and not the virus per se) [121]. Since CD8 T cells produce IFNγ, which is elevated in serum of IM patients [104,106-108,110] and IFNγ treatment can result in headache, fatigue, rigors, flu-like symptoms, and myalgias [187,188], empirically, it could be argued that CD8 T cell production of high levels of IFNγ results in the symptoms classically associated with IM. However, unlike the previous study [130], we found that severity of illness also correlated with blood viral load and not oral virus ([48] and Fig. 2-9). Subsequently, we observed a previously undefined positive correlation between blood viral load and CD8 lymphocytosis that could be suggestive of a model where systemic viremia drives activation of antiviral CD8 T cells, and therefore plays a critical role in disease severity. While we do not refute the conclusion that IM is an "immunopathogenic" disease, we highlight that viral load in the blood, which was previously disregarded, is an important aspect of the pathogenesis of IM.

Further in agreement with this and similar to the previous study looking at NK cell numbers and severity of pharyngitis [106], we found a positive correlation between severity of illness and NK cell expansion. In contrast however, we showed here that NK cell expansion correlates positively (and not inversely, [106]) with blood viral load. There are several possible explanations for the differences between out studies and those of Williams et al. First, the interpretation of the data presented by Williams et al. is confounded by the fact that while NK cell numbers were examined in association with disease severity, NK cell frequency (i.e. percentages) were examined when comparing viral load in the blood. Secondly, Williams et al. represented viral load per $10^6$ PBMC [106] while we represented viral load as the log transformed value of EBV per milliliter of

blood. Because of the massive increase in CD8 frequency during acute IM, using NK cell percentages may not accurately reflect the actual changes in the expanding immunological compartment. While this remains a potential source of discrepancies in our observation, it is unlikely because in a subset of this cohort, we still observed a positive correlation with viral load in the blood and the frequency of NK cells, regardless of whether we looked at time of diagnosis or at peak expansion (data not shown).

More relevant may be the different ways in which peripheral viral load is represented. Our group has shown in the past that plasma and PBMC viral load are inferior indicators for host control of EBV infection in comparison to whole blood viral load [48]. The data herein is composed of a larger set of individuals with internal controls for NK cell numbers and frequency before, during, and after EBV infection. Therefore. It is likely to reflect a more accurate interaction between viral load in the blood, stimulation of NK cell expansion, and NK cell antiviral activity.

The findings of a positive correlation between the blood viral load and host response factors such as increased CD8 and NK cell numbers is novel but not surprising. However, it is inconsistent with the previous paradigm [121] that external pre-existing factors (e.g. memory T cells reactivity to heterologous viruses) control the CD8 T cell response and result in symptoms during primary EBV infection. Finally, in support of our modified model of EBV pathogenesis, serum cytokine analysis suggested a stronger correlation with innate response than adaptive immunity. Indeed, upregulation of granzyme B on CD8 T cells specific for a viral infection is consistent with a type I interferon effect [195], however further analysis of type I and II interferon responses may be warranted to understand how interferons contribute to both the acute and the latent phase of this persistent chronic infection.

Therefore, the data suggest that potential strategies for preventing IM could be aimed at reducing blood viral load, either directly via antiviral therapy or indirectly through vaccination (i.e. to optimize the efficacy of the immune response).

## Subjects, Materials and Methods

### Design of prospective study

We recruited healthy volunteers from the University of Minnesota undergraduate residence halls in 2006 and 2007 (n=546). EBV seronegative exposure status was confirmed by the lack of IgG antibodies against EBV viral capsid antigen (EBV VCA IgG) (n=206). Of the 206 eligible EBV-naïve subjects, 147 (71%) were enrolled in the prospective study. For enrolled participants, blood was collected at least every 8 weeks during the academic year in addition to an electronic monitoring journal to track development of symptoms between visits. Subjects with symptoms consistent with acute primary EBV infection were required/encouraged to come in to the clinical virology research clinic for both a physical exam and laboratory-confirmation of primary EBV infection (n=66). Healthcare service personnel made the initial diagnosis of infectious mononucleosis. Primary EBV infection was confirmed by the presence of IgM antibodies to VCA and/or IgG antibodies to VCA or EBV nuclear antigen1 (EBNA1) and the presence of EBV DNA in the blood and/or oral samples as previously published [48] . Laboratory diagnosis of primary EBV infection was key to capturing asymptomatic seroconversion. Subjects continued pre-scheduled follow-up visits after seroconversion. All participants gave informed consent and the University of Minnesota Institutional Review Board approved all protocols used.

### Sample collection and handling

Peripheral blood samples obtained from subjects via venipuncture and collected in 10ml purple-top EDTA Vacutainer® tubes (Fisher Scientific). 200μl of blood was used for DNA extraction and HLA typing. Peripheral blood mononuclear cells (PBMCs) were isolated by Accuspin™ System-Histopaque®-1077 (Sigma-Aldrich) density gradient centrifugation per manufacturer's instructions. PBMC counts were recorded post-ficoll

purification of whole blood during each collection and stored as detailed below. Once pelleted, cells were frozen in 1 x $10^7$ cells/ml aliquots in a cryopreservative solution containing 90% FBS and 10% dimethysulfoxide ([DMSO], Sigma-Aldrich). Samples were allowed to slowly freeze at -80°C overnight and then transferred to liquid nitrogen for storage until needed. Cells were rapidly thawed in a 37°C water bath, diluted to 10ml in RPNK media supplemented with 50U/ml benzonase (Novagen) (RPNK media: RPMI 1640 (Cellgro) supplemented with 10% FBS (Atlanta Biologicals), 2% Penicillin - Streptomycin (5000U/ml, 5000µg/ml respectively, GIBCO, Invitrogen) and 1% L-glutamine (29.2mg/ml, GIBCO)). Cells were then counted using a hemocytometer and divided into separate fractions for flow cytometry or RNA processing.

Participants were asked to gargle 10ml of RPMI media for oral wash samples. Oral samples were then split into 2 aliquots: one for EBV quantification in oral supernatant and one for EBV quantification in oral cell pellet. Oral wash cell pellet results are adjusted for qPCR volume and then by volume of oral wash used. DNA was extracted from 200µl of either whole blood or oral washings using QIAamp DNA blood minikit (Qiagen). An EBNA1 plasmid standard was used as control and all samples were run using ABI TaqMan Universal PCR Master Mix (Applied Biosystem) [48] .

**Peptide MHC class I tetramers reagents**

Commercial tetramers were acquired from Beckman Coulter: EBV $BMLF1_{259-267}$ (GLCTLVAML)-A*0201 ready to use. Biotinylated MHC-peptide monomers from the National Institute of Health (NIH) tetramer facility were obtained for the following: EBV $BRLF1_{109-117}$ (YVLDHLIVV)-A*0201, EBV $BRLF1_{147-155}$ (RVRAYTYSK)-A*03, EBV $BZLF1_{190-197}$ (RAKFKQLL)-B*08; EBV $EBNA3A_{325-333\_}$ (FLRGRAYGL)-B*08, $EBNA3A_{379-387}$ (RPPIFIRRL)-B*07, $EBNA3A_{603-611}$ (RLRAEAQVK)-A*03. Prior to use, we added PE-, or APC-streptavidin (Invitrogen) to the monomers at a 4:1 molar ratio for O/N in the dark

at 4°C to generate fluorescent pMHC tetrameric complexes. All tetramers were stored in the dark at 4°C.

**Flow cytometry analysis**

PBMCs from each subject were stained with antibodies (e-Bioscience, Biolegend, or Invitrogen) against CD3, CD4, CD8, CD56, CD38, HLA-DR and peptide-MHCI tetramers (NIH tetramer facility or Beckman Coulter), washed, permeabilized, and then stained with an antibody to Granzyme B using the BD Cytofix/Cytoperm Kit per manufacturer's instructions (BD Biosciences). Samples were analyzed on a LSR II (Becton Dickinson) and all data was processed using the FlowJo Software from Treestar.

**Plasma Cytokine Measurements**

For plasma collection, lab personnel centrifuged whole blood for 10 minutes. Once separated from the cellular components, a sterile serological pipet was used to create 0.5ml to 1ml aliquots. These were cryopreserved until later use. Concentrations of cytokines in plasma were determined with human multiplex luminex kits (Panomics) and read on a BioPlex 200 reader (BioRad). Samples prior to primary EBV infection were used as patients' baselines.

**Statistical Analysis**

All statistical analysis was performed using Prism software (Graphpad) or Excel (Microsoft). Comparisons between groups were performed with either an unpaired two-tailed *t*-test with a p value less that 0.05 as the cutoff for statistical significance. For correlations, a Spearman rank correlation coefficient (*r*) was calculated and linear regression line added for clarity.

# CHAPTER 3

## The persistence of CD8 memory T cells for heterologous viral infections during primary EBV infection in humans

This chapter subsequently published in *Journal of Experimental Medicine*, Vol 209, Mar 2012, p. 471-480; Odumade OA, Knight JA, Schmeling DO, Masopust D, Balfour HH Jr, Hogquist KA. Primary Epstein-Barr virus infection does not erode preexisting $CD8^+$ T cell memory in humans. doi: 10.1084/jem.20112401. 

Immunological memory is key to host defense against the multiple different pathogens to which humans are constantly exposed. In the previous chapter, we defined the kinetics of the CD8 T cell lymphocytosis that occurs during acute primary EBV infection. However, it was still possible that activation and expansion of bystander memory T cells contributed to this lymphocytosis. Furthermore, acute primary EBV infection results in an unusually robust CD8 T cell response in young adults, and thus would be predicted to result in attrition of pre-existing memory to other infections. In order to address these issues, we analyzed samples from a longitudinal prospective study of primary EBV infection in humans.

Because CD8 memory T cell attrition could be dependent on the presence or absence of cognate antigen, we examined memory CD8 T cells specific for both human cytomegalovirus (CMV) and influenza (Flu) antigens, respectively. Surprisingly, both CMV- and Flu-specific CD8 T cells showed signs of activation including upregulation of granzyme B, suggesting that primary EBV infection causes activation of bystander CD8 T cells. However, Flu- and CMV-specific T cells generally did not expand, suggesting that the profound CD8 lymphocytosis associated with acute primary EBV infection is composed largely of EBV-specific T cells. Finally, we did not observe attrition of CMV- or Flu-specific T cells, even during acute infection. In general, the data support the concept that a robust CD8 T cell response creates a new memory CD8 T cell niche without substantially depleting pre-existing T cell memory for heterologous infections.

## Introduction

Adequate immunological memory is the basis of secondary immunity and vaccination. Unlike naïve CD8 T cells that exist at low precursor frequencies, memory CD8 T cells exist at higher frequencies, which allows for faster responses to secondary infection. Therefore maintenance of memory CD8 T cells is an important protective aspect of the immune response. Furthermore, although there is some heterogeneity within the memory CD8 T cell populations, there is some overlap in the homeostatic requirements (i.e. cytokines) [196].

Under normal conditions, naïve T cells respond to their cognate antigen by undergoing multiple rounds of proliferation and differentiation, which allows for an effector response that aids in pathogen clearance. Classically, after the acute immune response, the bulk of the expanded CD8 population undergoes apoptosis and only a small fraction is recruited into the memory phase [197]. It is thought that this acute phase attrition is key to making room for future responses to heterologous infections.

Interestingly, it has been suggested that during sequential heterologous infections, pre-existing memory CD8 T cells specific for bacteria [198], parasites [199] and especially viruses [200,201] undergo attrition. Using lymphocytic choriomeningitis virus (LCMV), Selin et al. showed that subsequent heterologous infections with vaccinia virus (VV), pichinde virus (PV), murine cytomegalovirus (MCMV) and/or vesicular stomatitis virus (VSV) resulted in a reduced frequency [200], and total numbers [201] of LCMV-specific CD8 T cells in the spleen. Subsequent data looking at human CMV-specific CD8 T cells during the onset of acute hepatitis B infection [202] would suggest that a secondary response can outcompete the first. Put together, proponents of the attrition model would suggest that vaccines and robust CD8 T cell responses would result in detrimental gaps in the immunological compartment, resulting in the inability to control subsequent re-infections or viral reactivation.

Conversely, recent data published by Veyzs et al. suggest that attrition may not occur in the setting of vaccination because with each subsequent immunization in a prime-boost model, the number of pre-existing memory CD8 T cells was maintained [203]. Nonetheless, in the context of human immunology, where each individual is challenged with a broad variety of pathogens and adequate immunity is required for long term protection, deciphering the impact of heterologous infections on pre-existing memory T cells after an acute infection is essential.

In order to test this in humans, we chose to study the effect of primary Epstein Barr virus (EBV) infection on pre-existing memory CD8 T cells to influenza (Flu) and cytomegalovirus (CMV). As one of the eight known human herpesviruses, primary EBV infection results in an acute immune response, characterized by a robust CD8 T cells expansion followed by a latent, predominantly immunologically silent chronic infection [121]. Transmission occurs via respiratory secretions, saliva, or blood from an EBV-positive healthy individual to an EBV-naïve person. When primary infection is symptomatic, it causes infectious mononucleosis (IM), which is characterized by a sore throat, fever, hepatosplenomegaly, lymphadenopathy, and fatigue.

Peptide-MHCI tetramers (for Flu and CMV) and/or presence of IgG antibodies (for CMV) were used in order to establish prior immunity to Flu and CMV. We then analyzed both the phenotype, the frequency and total number of EBV-, Flu- and CMV-specific CD8 T cells before, during, and after acute infection with EBV in a longitudinal prospective study. Interestingly, while pre-existing memory cells became activated during acute primary EBV infection, we did not observe a reduction in total Flu- or CMV-specific CD8 T cells after IM was resolved, suggesting that in an heterologous EBV infection in humans, booster immunizations are not necessary to maintain pre-existing memory CD8 T cells.

In conclusion, we performed the first study of bystander memory CD8 T cell activation and attrition in healthy humans upon a natural primary viral infection and showed that each memory population studied had a niche independent of the other heterologous virus-specific CD8 T cells.

## Results

### Prevalence of CMV and HSV infection prior to primary EBV infection was low

We performed a longitudinal study that followed a cohort of 147 Epstein-Barr virus (EBV) naïve college students. 67 of these students acquired primary EBV infection naturally. Serum from whole blood was evaluated for EBV genomes by qPCR and for antibodies specific to EBV viral antigens. In order to determine the presence of pre-existing immunity to herpes simplex virus-1 (HSV-1) and human cytomegalovirus (CMV), serums samples were tested for virus specific IgG antibody responses. HSV-1 seroprevalence in United States college students is approximately 37.2% in first-year and 46.1% in fourth- year students [204], however; of the 67 subjects tested, none were seropositive for HSV-1 IgG. CMV seroprevalence on the other hand, was 21.1% (20/95) in this cohort, which is also lower than published rates [205].

To study existing immunity to an acute virus infection, we studied influenza (Flu). We limited the analysis to only individuals who were HLA-A2 positive (51% in the cohort) in order to use available peptide:MHCI (pMHCI) tetramers to establish pre-existing Flu memory T cells. In the 17 subjects analyzed (Table 3-1), all but one had CD8 tetramer+ T cells for the HLA-A2 Flu matrix 1 protein (GILGFVFTL). The outlier individual likely reflects a mismatch between this reagent's sub-allele and that of the subject. Put together, the data suggest a high prevalence of Flu-specific CD8 T cell memory.

**Table 3-1. Subject Characteristics**

| Variable | Subjects (n=24) |
|---|---|
| *Sex- no (%)* | |
| Male | 9 (37.5%) |
| Female | 15 (62.5%) |
| *Average Age- yr* | |
| Mean (Range) | 18.7 (18.1 – 20.3) yrs |
| *Clinic visits per subject - no* | |
| Mean (Range) | 15 (6 – 31) |
| *Prevalence of infections tested* | |
| CMV IgG+ | 12/24 (50%) positive |
| HSV IgG+ | 0/24 (0%) positive |
| Flu A2 Tetramer+ | 16/17 (94%) positive |

**Total CD8 T cells showed expansion and activation during acute infection**

To define the activation status of CD8 T cells before, during and after primary infection, we stained PBMCs with antibodies to granzyme B and to the T cell activation markers HLA-DR (DR) and CD38 [183,186]. As expected, we observed a marked increase in the frequency and number of total CD8 T cells in the blood during acute primary EBV infection (Fig. 3-1A and Fig. 3-2).

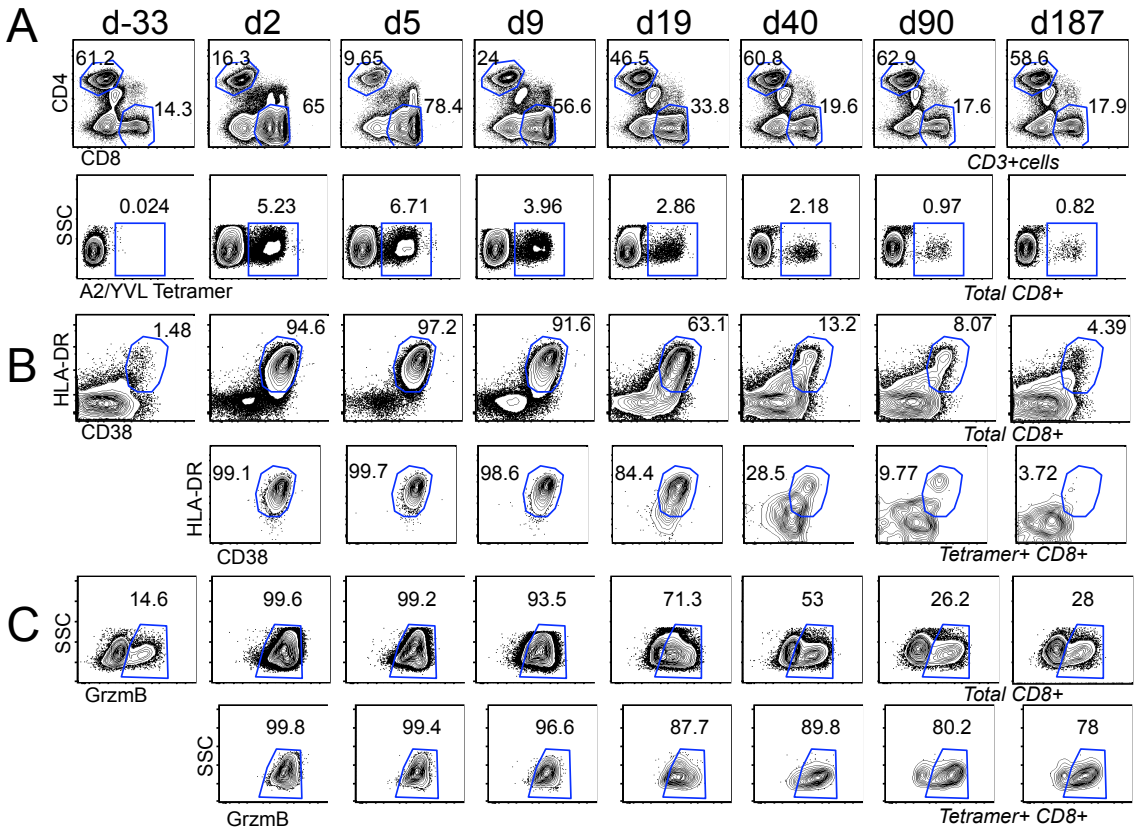


**Figure 3-1. EBV-specific T cells showed a classic expansion/activation pattern during acute infection**. Sample multi-parameter flow cytometric analysis of PBMCs from one subject prior to, during and after acute infection with EBV. (A) Top: CD4 and CD8 expression on CD3+ T cells over time. Bottom: CD3+ CD8 T cells specific for the HLA-A2/YVL epitope identified using pMHCI tetramers. (B) Dot plots showing activation markers, CD38 and HLA-DR (DR), expression over time on total CD3+ CD8 T cells (Top) and Tetramer+ CD3+ CD8 T cells specific for HLA-A2/YVL (Bottom). (C) Dot plots showing granzyme B expression over time on total CD3+ CD8 T cells (Top) and CD3+ CD8 tetramer+ T cells specific for HLA-A2/YVL (Bottom).

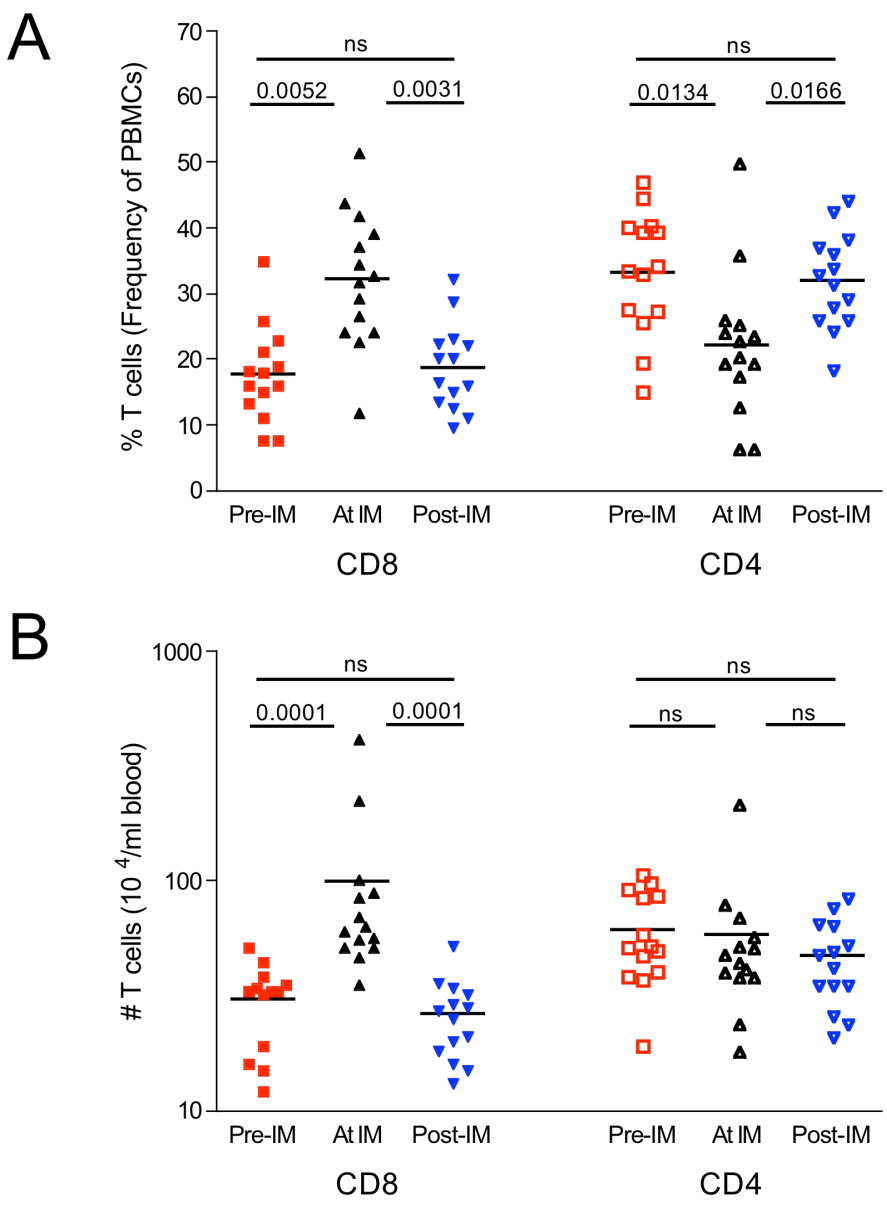


**Figure 3-2: CD8 T cell frequency and numbers are increased during primary EBV infection but CD4 T cell numbers are not.** PBMCs were stained with antibodies against CD3, CD4, and CD8. (A) The graph represents the frequency of CD3+ CD8 T cells or CD3+ CD4 T cells prior to (red square), during (black triangle), and after (blue inverted triangle) acute primary EBV infection. (B) To calculate total cell numbers, the frequency of CD8 or CD4 T cells relative to the live gate was calculated using FlowJo (Treestar) and then multiplied by the PBMC count per milliliter of blood. The graph represents the total number of CD3+ CD8 T cells or CD3+ CD4 T cells prior to (red square), during (black triangle), and after (blue inverted triangle) acute primary EBV infection. Wilcoxon signed rank test, $p<0.05$ considered statistically significant. (n=14)

In this subject population, we observed average baseline expression of 3.65% ±4.35 CD38+/DR+ and 13.7% ±8.49% granzyme B+ CD8 T cells prior to EBV infection (Fig. 3-1B/C, and Fig. 3-3). During acute infection, activation resulted in an average of 65.4% ±25.6% CD38+/DR+ and 74.1%±25.6% granzyme B+ CD8 T cells. In some individuals, this level approached 99% (Fig. 3-1, and Fig. 3-3). CD38, DR and granzyme B expression on total CD8 T cells returned to baseline around 150 days past acute illness (Fig. 3-1B/C, and Fig. 3-3).

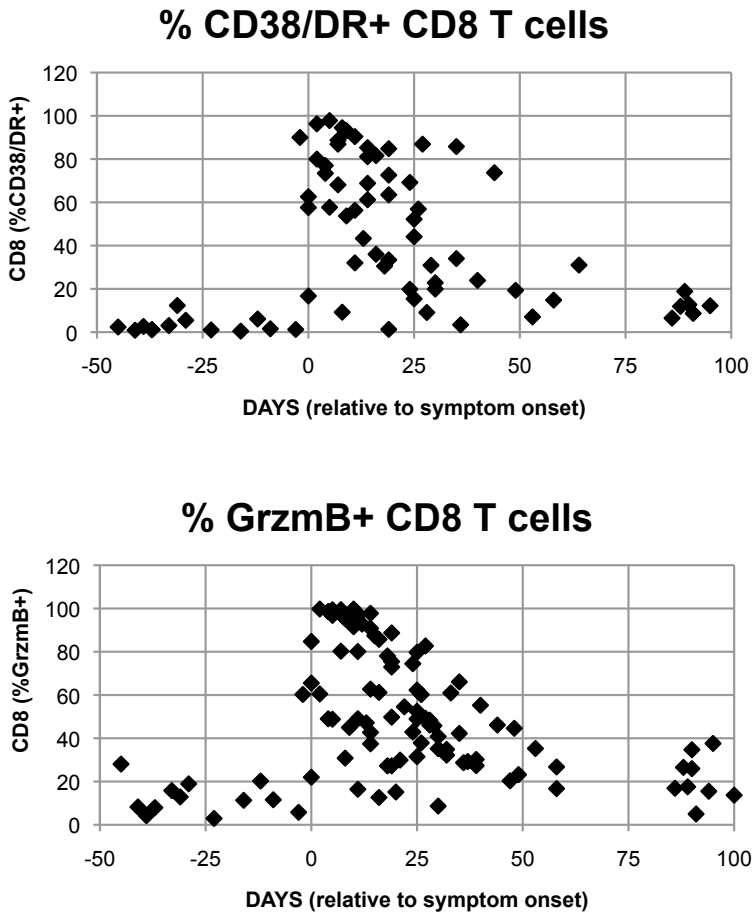


**Figure 3-3: Elevation of CD38/DR and Granzyme B on CD8 T cells.** PBMCs were stained with antibodies against CD3, CD8, HLA-DR (DR), CD38 and granzyme B. The graphs represent the frequency of CD38/DR+ CD8 T cells (top) or granzyme B+ CD8 T cells (bottom) over time relative to onset of symptoms (n=24)

The robust activation and expansion of total CD8 T cells during acute primary EBV infection has been suggested to be largely or entirely composed of EBV-specific T cells [121]. To test this, we evaluated CD8 T cells specific to EBV and other viruses using pMHCI tetramers (Fig. 3-1A). EBV-specific T cells expressed high levels of CD38, DR, and granzyme B during acute infection, as expected (Fig. 3-1B/C and Fig. 3-4B black symbols). This was observed with both lytic and latent antigens and was consistent between individuals with different MHC alleles (data not shown). The level of expression of activation markers on total CD8 T cells returned to baseline more rapidly than on EBV-specific T cells (Fig. 3-1B/C). This discrepancy might reflect different kinetics between CD8 T cells specific for different viral epitopes. Alternatively, it might reflect different kinetics between CD8 T cells specific for EBV versus non-EBV epitopes, since it has been reported that IFN can cause T cell activation, independent of antigen specificity [195,206]. Thus, we considered the possibility that activation of CD8 T cells specific for non-EBV antigens also occurred during primary EBV infection.

**Activation of pre-existing bystander memory CD8 T cells during acute IM**

We used pMHCI tetramers to detect T cells specific for Influenza (Flu) and cytomegalovirus (CMV) to determine the activation status of non-EBV memory CD8 T cells. To ensure we were evaluating pre-existing memory CD8 T cells and not co-infection, for CMV, we analyzed individuals who were CMV seropositive prior to EBV infection. For Flu, we selected individuals that had a readily detectable population of tetramer-positive cells that displayed a “memory” phenotype in samples taken at least 60 days prior to primary EBV infection. When we gated on the tetramer+ population prior to EBV infection, we observed relatively low levels of activation markers on Flu-specific and CMV-specific memory T cells, as expected (Fig. 3-4). Nonetheless, CMV-specific T cells tended to express more granzyme B (and DR) at baseline than Flu-specific T cells (Fig.

3-4). Interestingly, both Flu- and CMV-specific T cells showed upregulation of CD38, DR, and granzyme B during acute primary EBV infection (Fig. 3-4). In some cases, this activation occurred at levels nearly equivalent to those of EBV-specific T cells. By day 150, the proportion of activated Flu- and CMV-specific CD8 T cells had returned to baseline (Fig. 3-4). Thus, the data clearly demonstrated "bystander activation" of CD8 T cells during acute primary EBV infection.

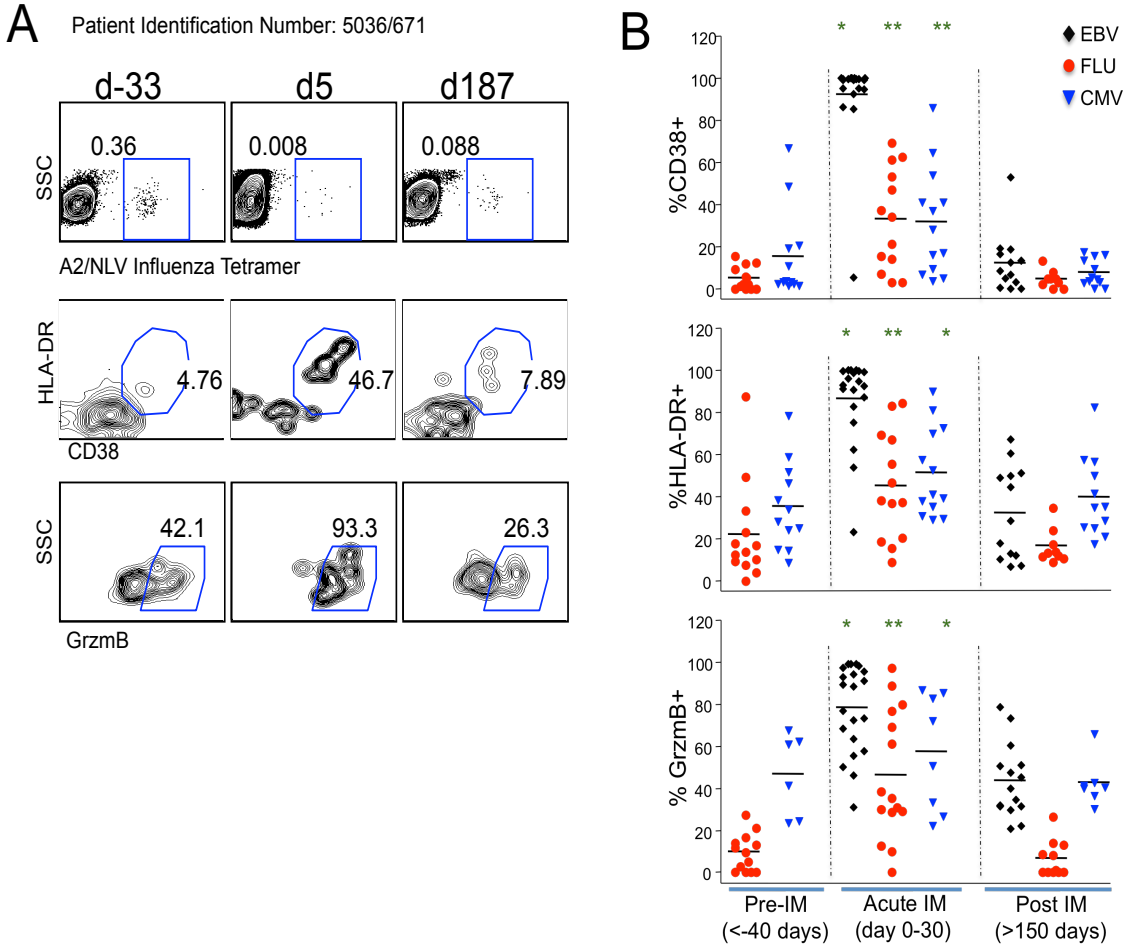


**Figure 3-4. CMV- and Flu-specific CD8 T cells became activated during acute primary EBV infection.** PBMCs from each subject were stained with antibodies against CD3, CD4, CD8, CD38, CD45RA, DR, pMHCI tetramers, and granzyme B. Percent of tetramer+ CD3+ CD8 T cells that expressed CD38 (top), DR (middle), and granzyme B (bottom) prior to (>40days prior), during (0-30 days), and after (>150days) acute primary EBV infection. EBV-specific CD8 T cells were identified with HLA-A2/YVL, or A3/RVR, or B8/RAK tetramers; Flu-specific CD8 T cells were identified with HLA-A2/GIL tetramers; and CMV-specific CD8 T cells were identified with HLA-A2/NLV, B7/TPR, B8/ELR or B35/IPS tetramers. (n=6-19 per virus-specific T cell, p value (ANOVA) *= p<0.05**=p<0.001)

**Flu- and CMV-specific CD8 T cell numbers were unchanged or increased only slightly during acute primary EBV infection**

From the data, it appeared that T cells specific for other viruses became activated during primary EBV infection. However, it was unclear if such cells expanded and contributed to the CD8 lymphocytosis that characterizes acute primary EBV infection [121], or underwent apoptosis [201]. To address this, we calculated the total numbers of Flu- and CMV- antigen specific CD8 T cells per milliliter of blood before, during, and after acute primary EBV infection. Although we typically observed reduced frequency of Flu- or CMV-specific T cells during acute primary EBV (Fig. 3-4A and Fig. 3-5), the total number of Flu-specific T cells generally remained steady or slightly increased (Fig. 3-6).

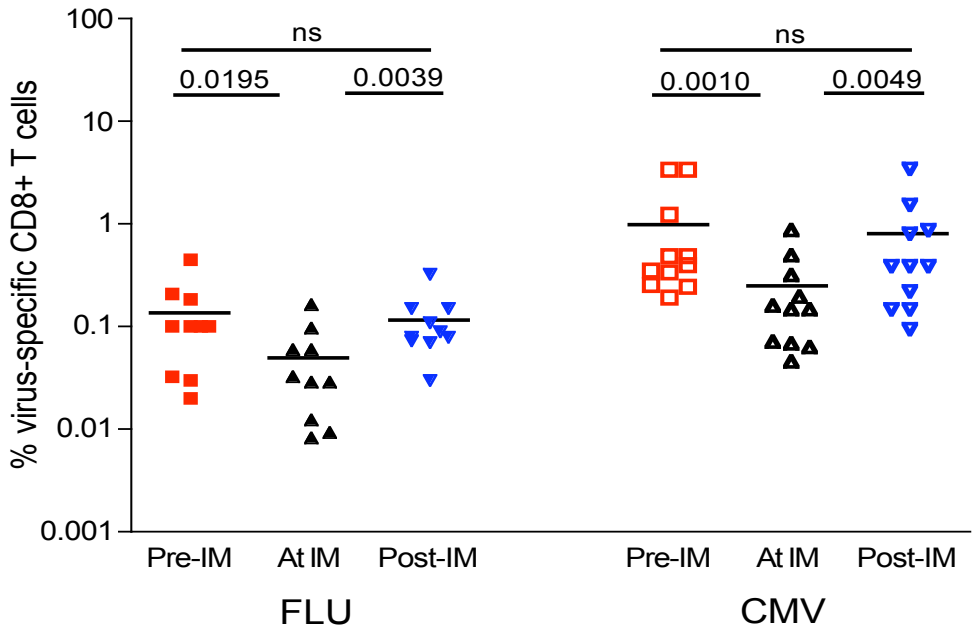


**Figure 3-5: There is a transient reduction in the frequency of pre-existing memory CD8 T cells during primary EBV infection.** PBMCs were stained with antibodies against CD3, CD4, CD8 and pMHCI tetramers. The graph represents the frequency of Flu (left) and CMV (right) tetramer+ CD3+ CD8 T cells prior to (red squares), during (black triangles), and after (blue inverted triangles) acute primary EBV infection. (n=10 for Flu, n=11 for CMV). Paired student *t*-test, $p<0.05$ is considered significant.

A modest but transient increase in the number of Flu-specific T cells (Fig. 3-6A) was observed in 4 of 15 individuals, and of CMV-specific T cells (Fig. 3-6B) in 2 of 12 individuals studied. The other individuals typically showed no increase or decrease of Flu-specific (Fig. 3-6C) or CMV-specific (Fig. 3-6D) CD8 T cells during after primary EBV infection.

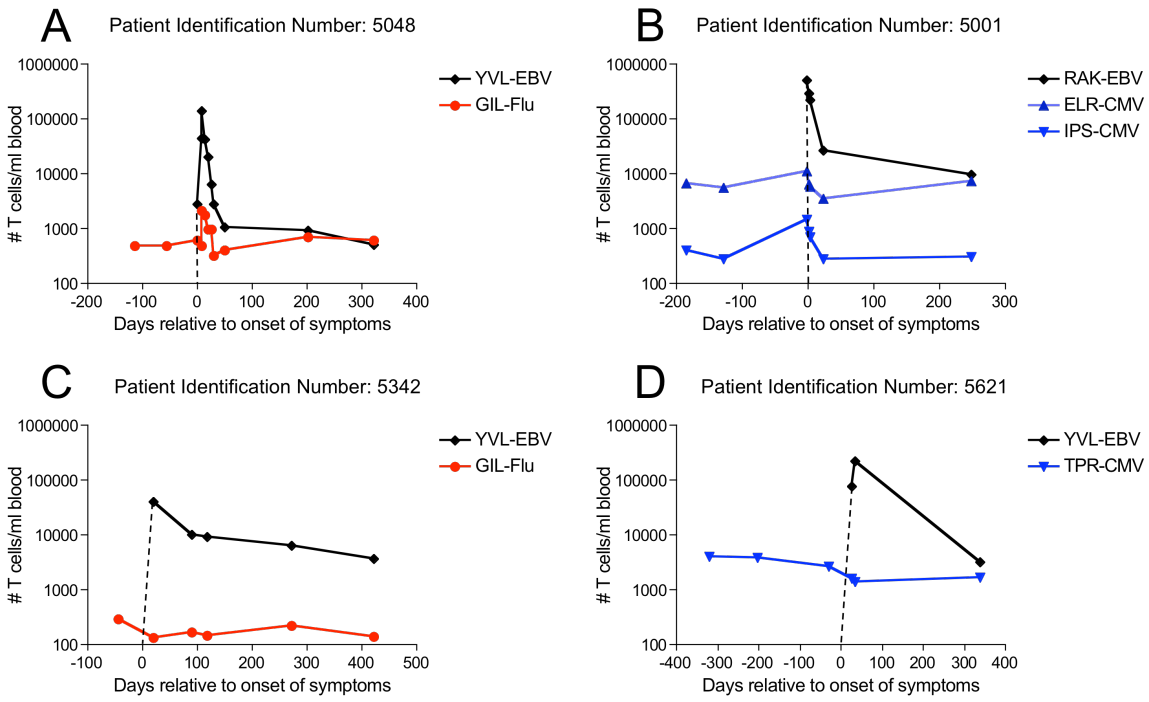


**Figure 3-6: Flu- and CMV-specific CD8 T cell numbers were unchanged or increased only slightly during acute primary EBV infection**. To calculate total cell numbers, the frequency of CD3+ CD8 tetramer+ cells relative to the live gate was calculated using FlowJo (Treestar) and then multiplied by the PBMC count per milliliter of blood. The data are sample graphs depicting the total T cells per ml of blood for EBV (black diamond), Flu (red circle), or CMV (blue upside-down triangle) over time in which non-EBV specific T cells either increased slightly (top panels) or stayed relatively unchanged (bottom panels).

To determine the relationship between bystander activation and T cell numbers, the number of Flu- or CMV-specific T cells per milliliter of blood during acute primary EBV infection was normalized relative to the pre-existing virus-specific cell numbers (set to 1) for each individual and this value was designated as fold expansion. When we

separated the subjects into two groups based on whether Flu/CMV-specific T cells underwent more bystander activation (>30% CD38/DR+) or less (<30% CD38/DR+), we found that the cells that underwent a greater extent of bystander activation showed a modest but significant increase in total numbers of bystander CD8 T cells in peripheral blood (Fig. 3-7). These data suggest that memory CD8 T cells specific for other acute (influenza) or chronic (CMV) viruses undergo bystander activation during primary EBV and may contribute to the increase in CD8 T cells numbers in the blood, but only slightly.

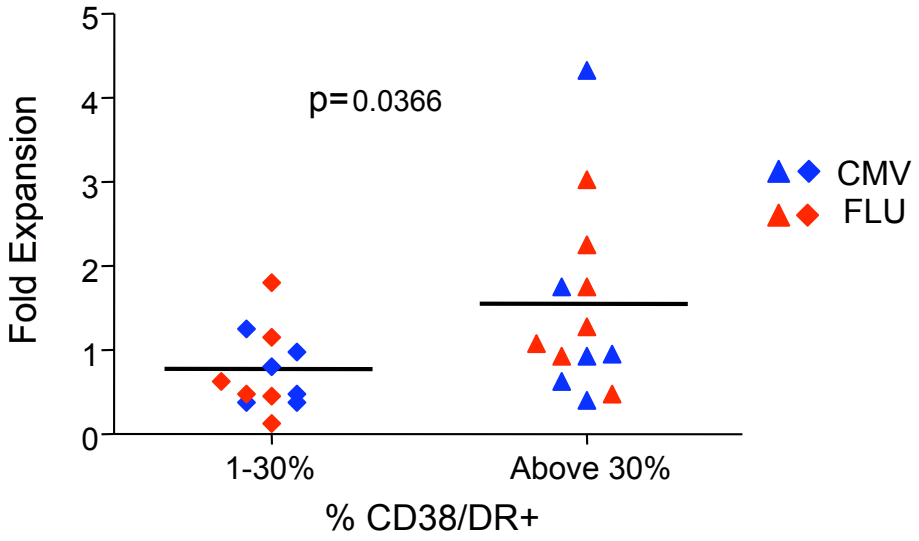


**Figure 3-7: High levels of activation during acute primary EBV infection were associated with modest bystander expansion**. Graph compares the fold expansion of CMV- and Flu-specific CD8 T cells that displayed an activated phenotype (based on >30% expression of CD38 and DR) or did not (1-30% expression of CD38 and DR). student *t*-test, $p<0.05$. (n=26)

Finally, we compared both the frequency and the numbers of bulk CD8 and CD4 T cells and antigen-specific (Flu- and CMV-specific) T cells prior to EBV exposure to those after resolution of the acute phase of infection. As shown in Fig. 3-2, although acute primary EBV infection transiently alters the CD8 T cell compartment, both the frequency and total numbers of CD8 and CD4 T cells did not significantly differ from baseline indicating that homeostasis of the peripheral immune compartment is not

compromised by infectious mononucleosis. Next, we explored whether attrition of pre-existing memory cells that were activated in a bystander fashion occurred. We compared both frequency (Fig. 3-5) and numbers (Fig. 3-8) to account for malleability in the CD8 T cell compartment. Surprisingly, there was no significant loss of either CMV- or Flu-specific T cells at later time points after EBV infection (Fig. 3-3, Fig. 3-5 and Fig. 3-8). Altogether, these data suggested that there is no attrition of peripheral blood memory CD8 T cells during or after heterologous infection in young adult humans.

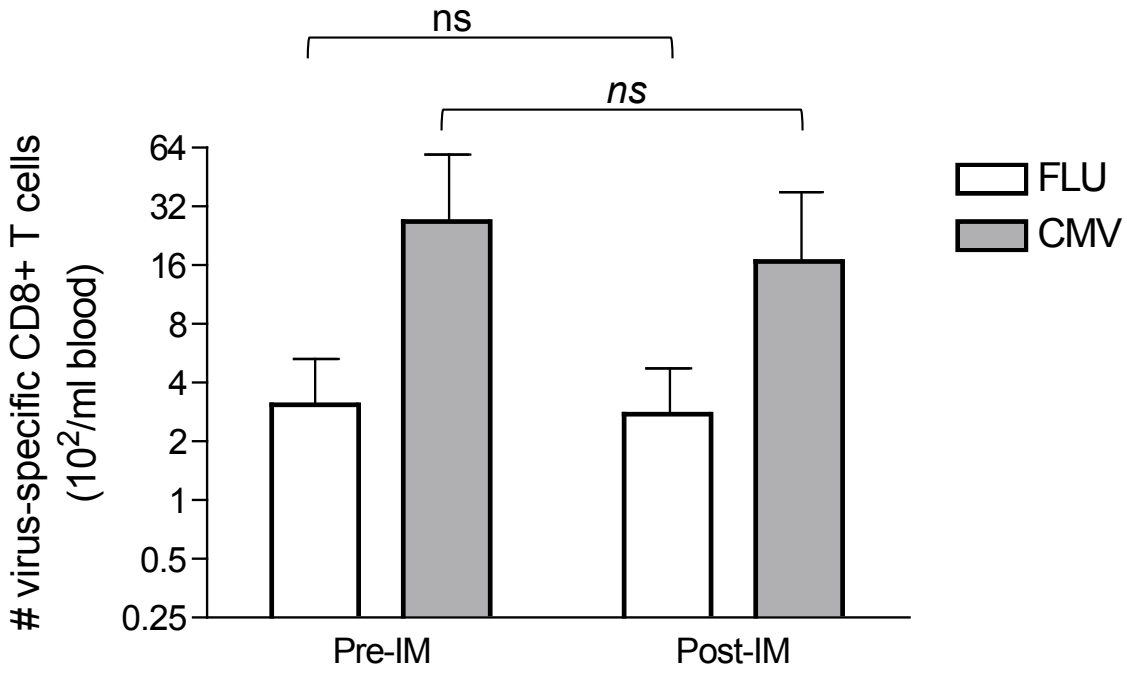


**Figure 3-8: There is no attrition of pre-existing memory CD8 T cells after primary EBV infection.** Total cell numbers for Flu-and CMV-specific CD8 T cells after 150 days post symptom onset were calculated as described above and compared to prior to EBV infection. The white bars (Flu) and gray bars (CMV) depict that the total virus specific CD8 T cells per ml of blood do not change significantly after infectious mononucleosis. P value of paired student *t*-test, $p>0.05$ (n= 10 for Flu; n=11 for CMV)

## Discussion

### Bystander activation during acute primary EBV infection

Heterologous infections can result in bystander activation of pre-existing memory T cells. In this study, we showed that bystander CD8 T cells undergo activation during Epstein Barr viral infection. Interestingly, we observed activation (increased expression of DR, CD38 and GrzmB) for CD8 T cells specific to both influenza (a cleared virus) and CMV (a latent virus). Since the reservoir for CMV includes monocytes, lymphocytes, dendritic cells and bone marrow cells [207], it is possible that the activated phenotype of CMV-specific T cells might reflect alterations in the viral load of CMV (i.e. viral reactivation) during primary EBV infection. Although we observed no evidence for this, the possibility is difficult to rule out due to the many depots for CMV besides peripheral blood. Furthermore, alterations in viral load cannot explain the activated phenotype of Flu-specific T cells. Put together, this suggests that the presence of cognate antigen is not necessary for bystander activation of memory T cells during an acute viral infection.

A few studies in humans have also suggested that bystander T cell activation can occur during chronic viral infections. Doisne et al. [185] showed that EBV-, CMV-, and Flu-specific cells exhibited a more activated phenotype (as assessed by DR and CD38) in patients with acute HIV infection compared with uninfected controls. More recently, activation of CMV- and EBV- but not Flu- specific cells was observed during acute hepatitis B infection [184]. In both studies, the small numbers of patients were not directly compared to controls [184] or to samples taken prior to infection [185]. Nonetheless, it would seem that activation of memory T cells specific for heterologous acute or chronic viral pathogens is a common occurrence during acute viral infection.

Both type I IFNs, and IL-15 have been suggested to mediate bystander activation in mouse studies [195,206,208]. Both cytokines were also shown to upregulate activation markers on human memory CD8 T cells when given at high concentrations *in vitro* [184,195].

Type I IFNs upregulated granzyme B in Flu-specific T cells [195] and IL-15 upregulated DR and CD38 on Flu, CMV, and EBV memory T cells [184]. Furthermore, from mouse studies, it has been postulated that production of both IFN$\alpha$ and IFN$\gamma$ can lead to activation of STAT1, subsequent production of IL-15 and IL-15R$\alpha$, culminating in bystander activation of CD122$^{int/hi}$ expressing memory T cells [209]. Therefore, the contribution of each cytokine to bystander T cell activation may not be exclusive.

Although IFNs have been detected in serum/plasma of infectious mononucleosis patients [104-110], IL-15 serum levels were not elevated during acute infectious mononucleosis [106]. However, *in vitro,* EBV induced IL-15 production in cultured peripheral blood mononuclear cells (PBMCs) [210]. Notably, this could be because non-hematopoietic cells might be a relevant source for this cytokine, so it is difficult to make conclusions about its potential role in bystander activation during primary EBV infection.

Since it is difficult to detect certain cytokines *ex vivo,* one potential approach has been to test the impact of each cytokine by looking at gene expression changes. Both acute viral infections [211] and vaccines [212] have been documented to generate a type I IFN gene signature. Preliminary data from microarray analysis of PBMCs suggest that a strong type I IFN and type II IFN response occurs during acute primary EBV infection, although IL-15 gene expression was not upregulated during acute primary EBV infection (Dunmire et al., unpublished data). Therefore, the gene expression signature during and after infectious mononucleosis still needs to be characterized to elucidate the mechanism(s) of bystander activation.

Furthermore, it is necessary to clarify the role that viral load may play in cytokine levels. In HIV infected individuals, activation markers on EBV-, CMV-, and Flu- specific T cells were higher than controls, and this was reduced by antiviral therapy [185] implying that viral load may induce the cytokines that cause bystander T cell activation.

Considering the potential significance of bystander activation (discussed below), if viral control is key, developing appropriate antiviral therapies is of utmost importance.

**Functional significance of bystander activation**

Despite the potentially common occurrence of bystander T cell activation in acute infection in humans ([184,195] and this study), it is unclear what the biological significance is. For example, the observation of bystander activation of CD8 T cells during primary EBV raises the question as to whether the extraordinary CD8 lymphocytosis observed in acute infection is composed entirely of EBV-specific T cells as previously suggested [186], or includes bystander T cells. Addressing this question with pMHCI tetramers has not been possible because of the large size of the EBV genome [121], and thus the large number of potential antigens that CD8 T cells may respond to. In this study, we observed an increase in the total numbers of CMV- and Flu- specific T cells during acute primary EBV infection, however this was only in some individuals, and was modest (even in the most highly affected individuals, <2-fold on average). This was compared to the 5-fold increase in total CD8 T cell numbers in the most highly affected individuals. In general, given the high frequency of memory CD8 T cells in adults (~50%), if all of them underwent even modest expansion, bystander T cells might comprise between 5-10% of the increased peripheral blood CD8 T cells during acute infectious mononucleosis, at least in the most affected individuals.

Moreover, since the precise cause of symptoms during acute primary EBV infection remains unknown, it is possible that bystander activation may contribute to immunopathogenic or protective aspects of the immune response. While Doisne et al. have suggested that bystander activation is of no consequence to HIV disease pathogenesis [185], it is possible that activation of bystander T cells could contribute to the development of clinical symptoms and/or could exacerbate the severity of IM during

primary EBV infection. Indeed, the severity of IM did correlate with the extent of CD38/DR or GrzmB upregulation on total CD8 T cells (see Chapter 2, unpublished observation). Based on the suggested mechanisms for upregulation of these activation markers [184,195], it would be interesting to see if IL-15 and/or type I IFN induced gene changes correlate with disease severity.

In terms of disease pathogenesis, the limited data correlating immune and viral parameters to disease suggest that lymphocytosis is key [121]. Therefore, high levels of cytokines produced by both EBV specific and bystander T cells may cause the symptoms of acute infectious mononucleosis. This might explain why adults, who are more immunologically experienced than children, experience more severe symptoms in response to acute primary EBV infection. However, we did not observe any difference in disease severity between subjects who were CMV infected prior to EBV versus those not (CMV-negative= 2.98$\pm$1.55; CMV-positive= 2.79$\pm$1.48; p= 0.68). Nonetheless humans bear many chronic viral infections by the time they reach adulthood [179], thus the contribution of a single one might have little impact.

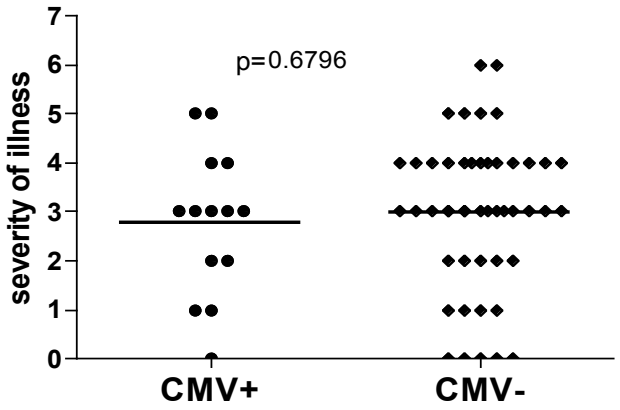


**Figure 3-9: CMV pre-infection did not alter disease severity during primary EBV infection.** The graph represents the severity of illness in individuals who were CMV seropositive (circle) or CMV seronegative (diamonds) prior to EBV infection. (n=14 and n=46, respectively). $p<0.05$ considered statistically significant for unpaired student *t*-test.

We document here the bystander activation of pre-existing memory CD8 T cells. However, it is possible that naïve, or even tolerized (self-antigen specific) T cells may also undergo bystander activation during primary EBV infection. Furthermore, it remains to be determined if bystander memory CD4 T cells become activated to a similar extent as CD8 T cells. Bystander activation of naïve T cells has been observed in animal models,[213] therefore, further studies will be required to address this potentially important issue in humans.

**Persistence of pre-existing memory CD8 T cells**

This analysis showed that pre-existing memory CD8 T cells specific for Flu or CMV did not wane during the course of primary EBV infection in humans. This was surprising since studies in animal models showed attrition of pre-existing memory CD8 T cells during many heterologous infections [198-201,214-218]. Further studies showed that both type I IFN and type II IFN can cause memory T cell attrition. In the bacterial model, attrition was dependent on IFN$\gamma$ [218], whereas in viral studies, bystander activation was dependent on Type I IFN and accompanied by apoptosis [206,213,219]

These findings may be at odds with these other studies for several reasons. First, it may indicate that EBV is not a strong IFN inducer, as some of the studies in animal models employed poly IC, an extremely potent inducer of type I IFN [206,213,219]. However, we find this unlikely because elevations in type II IFN [104,106-110], and less consistently type I IFN [104-106,109] have been detected during acute primary EBV infection. Furthermore, preliminary microarray data demonstrated a strong type I and type II IFN gene signature during acute primary EBV infection (Dunmire et al., unpublished data), suggesting that EBV would be a prime candidate for active depletion of pre-existing memory T cells.

Secondly, there is some evidence that central memory T cells (more likely to reside in lymphoid organs) are more sensitive to IFN induced apoptosis than effector memory T cells (more likely to circulate in blood) [220]. In this human study, we measured T cell numbers in the blood, in contrast to most animal studies, which focus on secondary lymphoid organs. Therefore, although there are studies that indicate attrition is a comprehensive process that occurs in both lymphoid and peripheral tissues [217,218], it is possible that depletion of pre-existing memory cells occurs in the main reservoir of viral infection (i.e. lungs, for influenza).

Finally, this analysis focused on heterologous infection in 18-21 year olds (mean 18.7) (Table 3-1). Based on the mouse study of LCMV Clone 13 versus LCMV Armstrong suggesting that memory T cell loss is a continuous process in persistent infections [217], the potential impact of EBV, as a latent chronic virus, on pre-existing memory may require longitudinal studies over decades.

On the other hand, these results are consistent with the work of Vezys et al., who showed using a prime-boost immunization model, that the CD8 T cell compartment expanded without depleting pre-existing memory T cell [203]. However, key to this observation was that using a prime-boost model prevented attrition of the LCMV-specific memory CD8 T cells [201]. In agreement with this, attrition of *Plasmodium berghei*-specific CD8 T cells after heterologous infection with LCMV, *Listeria monocytogenes*, vaccinia virus and mouse hepatitis virus 1 was reversed by booster immunizations [199]. Furthermore, in concert with data demonstrating that attrition is less pronounced in older mice than younger mice [219,221], it is possible that attrition will be observed only in the context of co-infection or serial heterologous infections.

In summary, we find that bystander activation of pre-existing memory T cells did not result in active depletion during a heterologous infection of EBV in humans. Because infection with mouse gammaherpesvirus 68 (MHV-68), the mouse model for

EBV infection, has been shown to result in partial depletion of pre-existing Flu-specific T cells, [216] these results emphasize the importance of studying immunity in both humans and animal models. Moreover, using a prospective study design, we provide a study protocol example that can be used in the future to evaluate current vaccination schemes such as measles, mumps, rubella (MMR) that challenge the immune system with multiple pathogens at once, and may therefore alter pre-existing immunological memory.

## Subjects, Materials and Methods

### Design of prospective study

As described in Chapter 2, we recruited healthy volunteers from the University of Minnesota undergraduate residence halls in 2006 and 2007 (n=546). All participants gave informed consent and the University of Minnesota Institutional Review Board approved all protocols used. EBV seronegative exposure status was confirmed by the lack of IgG antibodies against EBV viral capsid antigen (EBV VCA IgG) (n=206). Of the 206 eligible EBV-naïve subjects, 147 (71%) were enrolled in the prospective study. ELISAs were used to determine presence of CMV IgG and HSV IgG antibodies in some individuals.

For enrolled participants, blood was collected at least every 8 weeks during the academic year in addition to an electronic monitoring journal to track development of symptoms between visits. Subjects with symptoms consistent with acute primary EBV infection were required/encouraged to come in to the clinical virology research clinic for both a physical exam and laboratory-confirmation of primary EBV infection (n=66). Primary EBV infection was confirmed by the presence of IgM antibodies to VCA and/or IgG antibodies to VCA or EBV nuclear antigen1 (EBNA1) and the presence of EBV DNA in the blood and/or oral samples as previously published [48]. All participants continued pre-scheduled follow-up visits after seroconversion.

### Sample collection and handling

Peripheral blood samples were obtained from subjects via venipuncture and collected in 10ml purple-top EDTA Vacutainer® tubes (Fisher Scientific). 200μl of blood was used for DNA extraction and HLA typing. Peripheral blood mononuclear cells (PBMCs) were isolated by Accuspin™ System-Histopaque®-1077 (Sigma-Aldrich) density gradient centrifugation per manufacturer's instructions. PBMC counts were

recorded post-ficoll purification of whole blood during each collection and PBMCs were stored as detailed below. Once pelleted, cells were frozen in 1 x $10^7$ cells/ml aliquots in a cryopreservative solution containing 90% FBS and 10% dimethysulfoxide ([DMSO], Sigma-Aldrich).

Samples were allowed to slowly freeze at -80°C overnight and then transferred to liquid nitrogen for storage until needed. Cells were rapidly thawed in a 37°C water bath, diluted to 10ml in RPNK media supplemented with 50U/ml benzonase (Novagen) (RPNK media: RPMI 1640 (Cellgro) supplemented with 10% FBS (Atlanta Biologicals), 2% Penicillin - Streptomycin (5000U/ml, 5000µg/ml respectively, GIBCO, Invitrogen) and 1% L-glutamine (29.2mg/ml, GIBCO)). Cells were then counted using a hemocytometer and divided into separate fractions for flow cytometry or RNA processing.

**Peptide MHC class I tetramers reagents**

Commercial tetramers were acquired from Beckman Coulter: Human CMV $pp65_{495-503}$ (NLVPMVATV)-A*0201, CMV $pp65_{417-426}$ (TPRVTGGGAM)-B*0702, CMV $pp65_{123-131}$ (IPSINVHHY)-B*3501, CMV $IE1_{88-96}$ (ELRRKMMYM)-B*0801, Influenza A Matrix-$1_{58-66}$ (GILGFVFTL)-A*02; EBV $BMLF1_{259-267}$ (GLCTLVAML)-A*0201 ready to use. Biotinylated MHC-peptide monomers from the National Institute of Health (NIH) tetramer facility were obtained for the following: EBV $BRLF1_{109-117}$ (YVLDHLIVV)-A*0201, EBV $BRLF1_{147-155}$ (RVRAYTYSK)-A*03, EBV $BZLF1_{190-197}$ (RAKFKQLL)-B*08; EBV $EBNA3A_{325-333\_}$ (FLRGRAYGL)-B*08, $EBNA3A_{379-387}$ (RPPIFIRRL)-B*07, $EBNA3A_{603-611}$ (RLRAEAQVK)-A*03. Prior to use, we added PE-, APC-streptavidin (Invitrogen) to the monomers at a 4:1 molar ratio for O/N in the dark at 4°C to generate fluorescent pMHCI tetrameric complexes. All tetramers were stored in the dark at 4°C.

**Flow cytometry analysis**

PBMCs from each subject were stained with antibodies (e-Bioscience, Biolegend, or Invitrogen) against CD3, CD4, CD8, CD38, CD45RA, HLA-DR, peptide-MHCI tetramers (NIH tetramer facility or Beckman Coulter), washed, permeabilized, and then stained with an antibody to Granzyme B using the BD Cytofix/Cytoperm Kit per manufacturer's instructions (BD Biosciences). Samples were analyzed on a LSR II (Becton Dickinson) and all data was processed using the FlowJo Software from Treestar.

**Statistical Analysis**

All statistical analysis was performed using Prism software (Graphpad). Comparisons between groups were performed with either an unpaired two-tailed *t*-test , a paired two-tailed *t*-test (Fig. 5 and Fig. 8) or a one-way ANOVA with a p value less that 0.05 as the cutoff for statistical significance.

# CHAPTER 4

## Discussion/Conclusion

**Discussion**

In contrast to the 18,000 articles published on "EBV", there are approximately 1500 articles in a Pubmed search for " EBV infectious mononucleosis". Much of the EBV field has focused on immune impairment and EBV-related tumorigenesis due to the morbidity and mortality that can occur. This is partly due to an early study that suggested that IM was associated with CD8 lymphocytosis and not viral load in the blood [130]. Furthermore, anti-viral medications have had limited to no efficacy in treating IM [154-158,160,222]. Therefore, the scientific field has, in general, accepted based on limited and circumstantial evidence that the viremia itself does not contribute to the immunopathologic symptoms observed during infectious mononucleosis [121].

This is in contrast to the widely accepted notion in immunology that pathogen load is an important determinant of disease outcome. To date, only one other study has examined disease severity and viral load [48]. Although this group did not measure cell-mediated immune parameters, they did observe a strong association with disease severity and viremia [48]. Hence, the issue of what factors are associated with severe disease in response to primary EBV infection is still controversial. Reconciling these studies is key to determining potential therapeutic targets for primary EBV infection and EBV-related sequelae.

The work presented in this thesis provided insight into the mechanisms of protective immunity that may be important during primary EBV infection. We began by using a prospective study model to understand the basic immune responses to a natural primary EBV infection in humans and to facilitate capture of asymptomatic seroconversion to primary infection. Based on published data, we expected the incidence of infectious mononucleosis to be approximately 25% [121]. However, to our surprise, our data revealed that primary EBV infection results in clinical symptoms in 90% of individuals. When we compared clinical score and duration of illness in a

prospective study to that of a clinical study, we found approximately a 50% increase in both parameters. Based on these data, we concluded that studies that recruit participants from health services result in a distorted view of primary EBV infection because they focus on the most severe arm of infectious mononucleosis subjects.

Using this methodology, Chapter 2 describes the first prospective study, to our knowledge, that extensively analyzes acute primary EBV infection. We began by defining the peaks of viral load in the blood and oral cavity, CD8 lymphocytosis and NK cell expansion. Interestingly, the peak of the immune response occurred within the first two 2 weeks after symptom onset. At the peak of response, a majority of the CD8 T cells displayed (and to a lesser extent CD4 T cells) an activation phenotype (CD38, DR, GrzmB). The contraction and memory phase of the immune response was observed in some individuals to start as early as the third week after symptom onset. Moreover, we could not detect virus in the blood or oral cavity in most individuals prior to onset of symptoms. Since the estimated incubation period for development of primary infectious mononucleosis in response to EBV infection is between 32-49 days [178], the data support a model in which acute EBV infection results in a highly robust but delayed T cell response more similar to chronic viral infections like HIV and Hepatitis [183]. Therefore, although there is a long incubation period before the T cell response is visible, EBV is not unlike other chronic viral infections.

The design of the prospective model then allowed us to address whether disease severity was associated with CD8 lymphocytosis and/or EBV viremia. Here, we found that viremia, CD8 lymphocytosis and NK cell expansion all correlated with disease severity. Viral infection can result in the activation of pathogen associated molecular patterns such as toll-like receptors (TLRs), and subsequent activation of the innate immune system. Furthermore, viral antigens can engage T cell receptors and drive antigen specific proliferation of the adaptive immune system. Therefore, if viremia is the

determinant of NK cell and CD8 T cell expansion, we expected to see a positive correlation. An association between viremia, but not oral viral load, and both NK and CD8 T cell expansion corroborated this. One advantage of a prospective study is that we have banked PBMCs from EBV-naïve stages of all our subjects. Therefore, one potential experiment would be to compare, *in vitro*, the immune response of multiple individuals to varying amount of copies of EBV. If we observed similar proliferation across multiple individuals that correlated with EBV copy number used as stimuli, then this would also support an important role for EBV.

Next, we attempted to determine if any cytokines correlated with disease severity. Interestingly only IL-6 correlated with disease severity. IL-6 is a pleiotropic cytokine that plays an important role in immune regulation (innate and adaptive responses) and inflammation [223]. IL-6 is both a pro- and an anti- inflammatory molecule, which may be produced early by endothelial cells, and transignalling by IL-6 suppresses neutrophilic immune responses in part by inducing apoptosis [224]. Furthermore, IL-6 in tissues also results in enhanced monocyte recruitment, and can skew T cell differentiation towards T-helper 2 and T-helper 17 [224]. Therefore, it is possible that we observed a reduced disease severity because IL-6 is skewing the immune response away from a T-helper 1 (IFN$\gamma$) response.

However, based on the mouse model of EBV, mouse gammaherpesvirus 68 (MHV-68), we hypothesize that IL-6, per se, is not as important as viremia. Similar to humans, IL-6 is elevated during MHV-68 infection[225], however, IL-6$^{-/-}$ mice infected with MHV-68, do not differ in viral load in the lymphoid tissues (spleen and lymph node), CD8 T cell lymphocytosis or cytokine production from wild-type mice [226]. Yet, we cannot assume based on the mouse data, that IL-6 is negligible during acute EBV infection; therefore, more extensive analysis may be needed to determine the mechanism and role of IL-6 in development of IM in humans.

The data presented here does not refute the contribution of CD8 lymphocytosis to disease pathology, as we observed a strong correlation between CD8 lymphocytosis and both disease severity and duration of illness. Instead, we highlighted the contribution of viral load in the blood to CD8 T cell (and NK cell) expansion. As stated earlier, one rationale for CD8 lymphocytosis is the recruitment and activation of bystander T cells from the pre-existing memory CD8 T cell repertoire. However, previous reports are at odds as to whether or not, all CD8 lymphocytosis is EBV-specific [186,227]. Furthermore, multiple studies have observed bystander activation of pre-existing memory T cells.

Thus, in Chapter 3, we defined the interactions between a robust immune response on the pre-existing memory CD8 T cell compartment in a chronic infection in humans. For the first time, we showed bystander activation of Flu- and CMV-specific T cells during primary EBV infection. While bystander pre-existing memory CD8 T cells in general did not expand, we did not perform exhaustive analysis to determine the specific contribution of pre-existing memory to the expanded CD8 T cell population for each individual. Therefore, put together with the multiple chronic infections that each individual has been exposed to, heterologous immunity [135,180] may still be, in part, contributory to the dramatic CD8 T cell expansion observed during acute primary EBV infection.

**From Bench, To Bench, To Bedside**

In Chapter 2, we insinuated that the symptoms of IM may be ameliorated using antivirals. Although one study showed a partial effect with valacyclovir [58], other studies have not [154-158,160]. Given that certain antiviral drugs effectively reduced viral loads [58], the lack of reduced clinical disease is presumably because IM is typically not diagnosed until a person seeks treatment due to persistent symptoms which results in delayed therapy

initiation relative to the onset of symptoms. Furthermore, based on a study showing that continuous valacyclovir treatment results in reduced EBV infected B cells over time in healthy carriers [228], it would be interesting to determine the effectiveness of reducing IM symptoms and duration using extended antiviral regimen that avoid the viremia rebound detected after a 14-day valacyclovir schedule [58].

Another potential way to reduce viremia or prevent IM would be to immunize EBV naïve individuals with an EBV vaccine [170]. Also known as EBV-induced membrane antigen, gp350 protein facilitates infection by binding to CD21 on B cells [12] and preliminary results of a gp350 subunit vaccine have been promising [124,125,127]. In general, gp350 vaccines have been immunogenic, eliciting anti-gp350 antibodies (including neutralizing antibodies) in most subjects 1 month after the final vaccination [124,125,127]. Moreover, gp350-specific cell mediated responses have also been induced during vaccination as demonstrated by inhibition of the outgrowth of transformed B cells [125] and the incidence of infectious mononucleosis was reduced (efficacy= 78%) in a small cohort [127]. However, in a transplant study to prevent lymphoproliferation in EBV naïve children, a three-cycle gp350 vaccine schedule did not reduce viral load [126] warranting the need for further development of gp350 vaccine protocols. Furthermore, it is key to characterize oral and whole blood viral load, CD8 T cell, CD4 T cell, and NK cell responses, in addition to the humoral immunity elicited by gp350 immunization in healthy individuals for complete understanding of immunity to EBV.

Strategically, if the aim is to modulate the CD8 T cell immune response during acute primary EBV infection, it is important to determine the impact of this chronic infection on the stability of pre-existing memory T cells. In Chapter 3, we showed that primary EBV infection did not result in attrition of memory CD8 T cells specific for Flu and CMV. However, we did not determine if attrition of naïve (both antigen specific and self-specific) CD8 T cells occur after IM. If primary EBV infection results in depletion of

naïve precursor CD8 T cells, the potential impact on the ability to recruit adequate naïve CD8 T cells precursors during subsequent challenges to the immune system will be important to study and understand. We could also determine if primary EBV infection resulted in attrition of virus-specific cells that do not undergo secondary challenge, such as in subjects vaccinated with the yellow fever vaccine. Our group will most likely perform these experiments in the future.

Overall, the data suggest that EBV viral load in the blood (i.e. viremia) is an important driving force for both innate (NK cell expansion, IL-6 production) and adaptive (CD8 expansion) immunopathologic responses that result in the development of infectious mononucleosis during primary infection. Furthermore, the data would support a model in which an initial inadequate control of viremia (and not local (tonsillar) infection) propagates a massive but sub-optimal T cell response, which results in prolongation of infectious mononucleosis symptoms. Hence, these results provide several insights into an acute cell-mediated response to a persistent chronic virus in humans.

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