Immunological responses during the incubation period and acute phase of naturally acquired primary Epstein-Barr virus infection

A Thesis

SUBMITTED TO THE FACULTY OF THE

UNIVERSITY OF MINNESOTA

BY

Samantha K Dunmire

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Dr. Kristin A Hogquist

July 2015

Acknowledgements

First and foremost, I want to thank Kris Hogquist, my mentor. I have tremendous respect and affection for her, both as a scientist and a friend. I could not have asked for a better mentor, and I'm grateful to have been one of her students. To Steve Jameson for both his insight and his feedback through countless lab meetings. Thanks to the other half of team EBV, Dare Odumade. Without you, I doubt this project would exist, much less in its current form. You are an awesome partner in crime and friend.

I would also like to thank our clinical collaborators, in particular Dr. Hank Balfour, for his assistance and support over the years, and his lab staff, Max, Jen, and Jenn. You have all been enormously kind and helpful. Thank you to the numerous undergrads who passed through the Balfour lab and helped me wrangle samples from the liquid nitrogen.

Thank you to the members of my committee: Bryce Binstadt, Dave Masopust, Emily Gillespie, and Marc Jenkins. Your advice and guidance on this project has been enormously helpful, and I'm grateful to all of you for taking the time to shape me into a better scientist.

To all of the members of the Jamequist lab, past and present, who are too numerous to name. You have all offered nothing but encouragement, support, and helpful feedback. I'd like to especially thank our lab manager, Jane Ding, a mouse weaner without peer, and my fellow student Kell "MF" Holzapfel, who endured many of the trials and tribulations of grad school with me from start to finish.

To all the MICaB students and members of the Center for Immunology who have assisted me with their time, effort and criticisms. You're such a large part of what makes this training environment great.

Finally, thank you to my family and friends. My grandparents, all four of you, for teaching me and inspiring a love of learning. To my brothers, Davis and Russell. Friends, you know who you are.

Grant Support

This work was supported by the National Institutes of Health (T32 AI007313) and a Doctoral Dissertation Fellowship through the University of Minnesota.

Dedication

For my brothers

ABSTRACT

Epstein-Barr virus (EBV) in a human herpesvirus. It infects about 90% of the human population, and is the main causative agent of infectious mononucleosis. The incubation period, the time between viral acquisition and onset of symptoms, is unusually long in patients presenting with infectious mononucleosis, lasting about six weeks. In addition to causing acute illness, there can also be long-term consequences as the result of acquisition of the virus, including nasopharyngeal carcinoma and lymphoma. Nevertheless, there remains a surprising dearth of knowledge regarding the establishment of and immune response to persistent EBV infection in its natural hosts, especially during the incubation period.

We sought to address many of these gaps by studying the incubation period, acute phase, and convalescence of undergraduates experiencing infectious mononucleosis during primary natural EBV infection in a cohort of prospectively studied volunteers. Particular attention was paid to the previously uncharacterized incubation period. Our findings have focused on understanding the immune response that occurs in young adults presenting with infectious mononucleosis, via gene expression changes as observed in peripheral blood mononuclear cells and innate and adaptive immune cells.

Using a systems biology approach we discovered that important gene expression changes occur during the immune response to primary EBV infection. A typical antiviral type I interferon response was not observed at onset of infectious mononucleosis symptoms, but rather up to two weeks prior. The gene

expression signature at symptom onset was dominated by cell cycle related genes, probably due to the CD8 T cell lymphocytosis, and type II interferon regulated genes. Interestingly, comparison of the EBV signature with other acute viral infections revealed very little similarity. The EBV signature showed the greatest similarity with hemophagocytic syndromes. This result is consistent with the view that infectious mononucleosis is an immunopathologic disease, and is supported by evidence that EBV can cause hemophagocytic lymphohistiocytosis.

As an extension of this work, we carefully examined changes in cellular phenotypes and population frequencies to determine if there were significant alterations to certain compartments during the response to primary EBV infection. We observed a type I interferon signature in a larger subset of study participants during the incubation period. This response was concurrent with the transition of virally infected B cells from the oral cavity to the blood, a decline in plasmacytoid dendritic cells from the circulation, and a polyclonal CD8 T cell activation. No EBV specific CD8 T cells activation was observed until the onset of infectious mononucleosis symptoms.

A major obstacle to understanding EBV related sequelae has been the lack of an efficient animal model for EBV infection, although progress in primate and mouse models has recently been made. Taken together, the data compiled in this thesis provide important first descriptions of the immune responses that occur during the establishment of a natural persistent infection in humans. Key future challenges are to develop protective vaccines and effective treatment regimens.

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Introduction

INTRODUCTION

Epstein-Barr virus (EBV) is a highly ubiquitous human pathogen, infecting up to 90% of the human population by the age of 35. Infection with EBV is more common in less developed nations, and evidence is emerging that the seroprevalence is declining among wealthier countries (1, 2). Summarized here is our current understanding of how EBV is transmitted and replicates, the prevalent immune responses that control initial infection, and steps toward the treatment and prevention of EBV related diseases.

Epstein-Barr Virus

EBV is a member of the herpesviridae family of DNA viruses, which all cause lifelong infection in their hosts. Generally, herpesviruses are species specific, with each virus usually only naturally infecting one species of host and are limited to mammals, bird and reptiles. Of the over 200 discovered members, only nine of these infect humans (3). These viruses are classified on the basis of similarities in morphological and genetic criteria. Morphologically, herpesviruses hold a number of features in common: (i) a linear double-stranded DNA core, varying in length between 124-295 kb; (ii) an icosohedral capsid made up of 162 capsomeres; (iii) a poorly defined tegument surrounded by a lipid envelope with viral glycoproteins (4).

The human herpesviruses can be divided into three different subfamiles: alpha, beta and gamma herpesviruses. These subfamilies form latent infections in distinct cell types, neurons, lymphocytes, and B cells, respectively (3). Alpha herpesviruses include herpes simplex virus (HSV) 1, HSV 2, and varicella zoster

virus (VZV). Beta herpesviruess include cytomegalovirus (CMV), and human herpesviruses 6A, 6B, and 7. Gamma herpesviruses include EBV and Kaposi's sarcoma virus. While alpha herpesviruses can sometimes infect a wider range of hosts, both the beta and gamma herpesviruses are limited to the order of the natural host for the virus in question (3). Of these viruses, human herpesvirus-6 is estimated to be the most prevalent, with more than 90% of the population infected. The least common is Kaposi's sarcoma virus, with as little as 2% of the population infected (5).

Two distinct genera, rhadinovirus (RDV) and lymphocryptovirus (LCV), comprise the human gamma herpesvirus subdivision. Across species they show a high degree of genomic structural similarity, with the exception of a small subset of nuclear proteins (6, 7). Epstein-Barr virus is a member of the LCV genus, specifically an Old World LCV (8). Several species of primates are naturally infected with LCVs that have high colinearity with the human tropic EBV (3). It is important to note that LCVs exclusively infect primates. The mouse virus murid herpesvirus-68 (MHV-68), which is commonly used to model EBV infection is actually an RDV (9), and thus is not an ideal pathogen for recapitulating the response to EBV.

Across populations, there are two types of EBV. The main differences between these types EBV infected individuals in the Western world tend more often to be infected by type 1 EBV than type 2 EBV (3, 10). Whether or not superinfection with both strains is possible in immunocompetent individuals

remains unknown, although both strains are readily detectable in the oral cavity of patients with human immunodeficiency virus (11, 12).

Routes of Transmission

Kissing is the major route of transmission of primary EBV infection among adolescents and young adults. This was elegantly documented by Hoagland's careful clinical observations (13) and confirmed many decades later by a prospective study at the University of Minnesota (14). Penetrative sexual intercourse has been postulated to enhance transmission (15), but we have found that subjects reporting deep kissing with or without coitus had the same risk of primary EBV infection throughout their undergraduate years (14).

The incubation period of infectious mononucleosis is approximately 6 weeks. Hoagland's clinical records suggested an incubation period of 32-49 days based on the dates of kissing episodes until the onset of infectious mononucleosis (13). A well-documented case was reported by Svedmyr et al. (1984) in which the kissing event occurred 38 days prior to onset of symptoms (16). Behavioral data from our medical history questionnaires collected during prospective studies are consistent with an incubation period of 42 days (Balfour et al. unpublished observations).

Besides deep kissing, primary EBV infection can also be transmitted by blood transfusion (17), solid organ transplantation (18), or hematopoietic cell transplantation (19), but these routes account for relatively few cases overall. Alfieri et al. (1996) used polymorphisms in the EBV *BAM*HI-K fragment length

and size polymorphisms in EBV nuclear antigen (EBNA) -1, -2 and -3 proteins to identify the specific blood donor responsible for transmitting EBV to a 16-year-old liver transplant recipient who subsequently developed infectious mononucleosis.

The way young children contract EBV is unknown. A reasonable supposition is that they are infected by their parents or siblings who are "carriers" of the virus and who intermittently shed it in their oral secretions (20). An extreme example of this is the very early acquisition of EBV among three distinct Melanesian populations whose infants have multiple caregivers that premasticate the babies' food (21)

Incubation period of EBV

The long incubation period of EBV continues to be poorly understood due to a lack of samples obtained between the time of infection and presentation with EBV related symptoms. During primary infection, viral replication is first detected in the oral cavity (14). The virus can infect tonsillar epithelial cells as well as B cells in the parenchyma of the tonsil (22). There may be a cyclic nature to the pattern of infection in the oral cavity as it has been shown in vitro that virus derived from epithelial cells has a much higher entry efficiency for infecting B cells and vice versa, resulting in a switched viral tropism depending on the cell type in which the virus replicates (23), discussed in detail below.

During the incubation period, the virus moves from the oral cavity to the blood. Previously, little was known about the kinetics or means of this transition, but this interesting period has been more carefully elucidated by the work

described in this thesis. Early infection in the oropharynx is shown in Figure 1-1. A type I interferon response was detected by gene expression profiling of peripheral blood approximately 2 weeks prior to symptom onset in some subjects experiencing primary EBV infection. Viral genomes can sometimes be detected in the peripheral blood as early as three weeks prior to symptom onset and consistently at least one week prior to illness (24), where it is probably maintained latently in resting memory phenotype B cells (25).

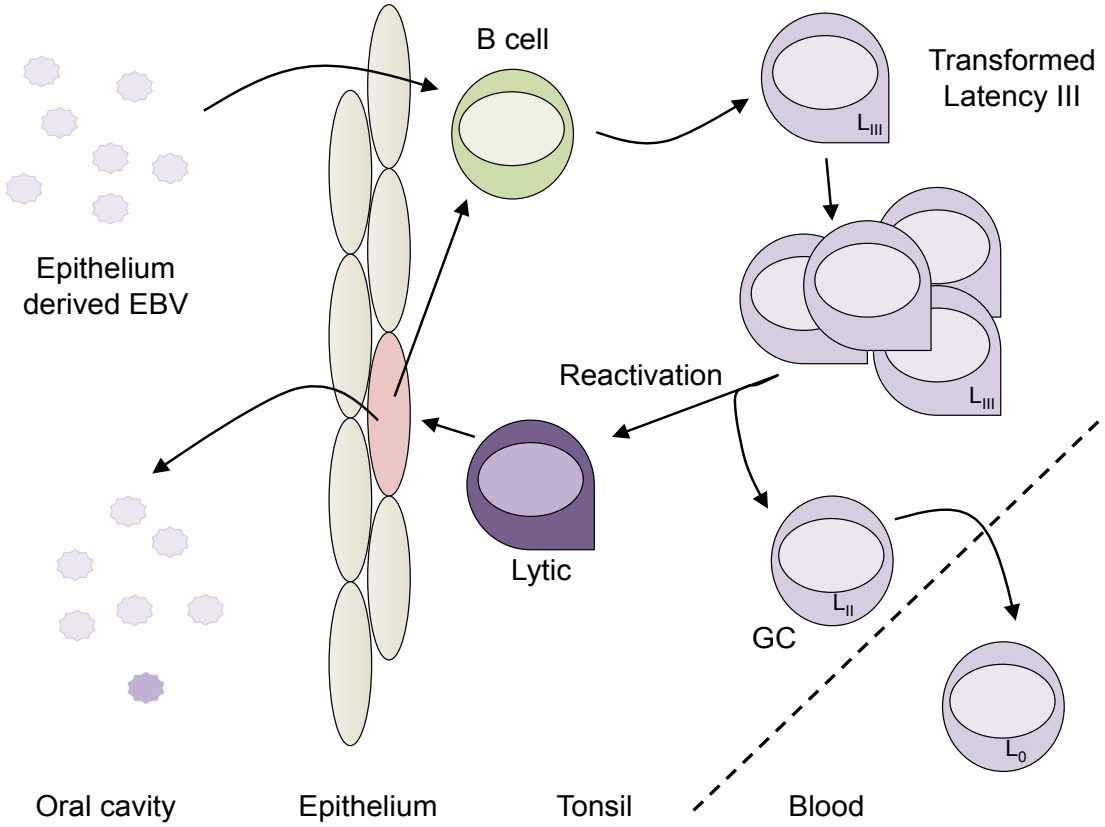


Figure 1-1. Establishment of initial EBV infection in the oropharynx and blood. Epithelial cell derived EBV is transmitted to an EBV naïve host via infected saliva. Viral particles infect susceptible B cells, resulting in transformed $Latency_{III}$ cells. These cells replicate, creating a reservoir of virus. Some pass through the germinal center reaction, with the latent membrane proteins augmenting B cell receptor signaling. These $Latency_0$ cells become memory-like, changing their trafficking patterns and recirculate between the periphery and oropharynx. Other $Latency_{III}$ cells undergo reactivation, producing virus which infects epithelial cells. Virus is then shed into the oral cavity.

Replication and Tropism

After an inoculum of saliva containing viable EBV enters the oral cavity of an EBV naïve host, the virus may infect either oral epithelial cells or B cells in the lymphatic tissue of Waldeyer's ring. While it is uncertain which of these events is more likely to occur first, attachment of EBV to B cells is effected by binding of

the EBV glycoprotein gp350/220 to the C3d complement receptor CD21 on B cells (26). Fusion and entry of the virus is then mediated by another EBV glycoprotein, gp42, which binds to the MHC class II molecule HLA-DR on the B cell to trigger endocytosis of the virus (27). Attachment to epithelial cells proceeds slightly differently. The EBV open reading frame BRMF2 encodes a protein that has binding affinity to a number of α integrins known to be present on surface of squamous epithelial cells (28, 29). In either case final fusion of EBV with the endocytotic membrane or plasma membrane, respectively, is mediated by gH/gL and gB (27). Virus may be replicated in either epithelial cells or B cells during persistent infection, although entry into epithelial cells is always significantly (logs) less efficient (23). Virus shed into saliva has been found to be primarily derived from epithelial cells due to the presence of gp42 and lack of HLA-DR present on the envelopes of EBV in saliva. These envelope protein differences obtained during budding explain the switch tropism observed in EBV entry patterns (30).

Once the viral genome has been transported to the nucleus, virally encoded DNA polymerases replicate the linear viral DNA. During the lytic phase of infection, there are three major phases of gene expression known as the immediate early, early, and late. Two key genes are expressed at the immediate early phase, BZLF1 and BRLF1. These genes encode the proteins ZEBRA and Rta, which are activators that initiate the transition from latency to lytic infection (31-33). Early phase genes generally consist of proteins important for the replication of the viral genome such as DNA polymerases, mRNA transport

enhancers, and factors for immune evasion. The late phase genes are largely structural proteins for packaging of the virus (3).

Latency is an important stage of the EBV life cycle, wherein latency is maintenance of viral infection without active lytic replication. Lytic replication is not the only mechanism that drives replication of the EBV genome. EBV genetic material may also be replicated during the various stages of latency through normal host DNA replication machinery. During latency, the EBV genome circularizes in the nucleus and is tethered to host chromosomes by the EBV nuclear antigen (EBNA)-1 protein (34). This episome contains an origin that is readily bound by host DNA polymerases in an EBNA-1 dependent fashion. EBNA-1 then assists in segregation of the replicated EBV genome to daughter cells following B cell division (3). This type of replication is considered “vertical” replication.

A number of latency stages exist for EBV infected B cells, some of which are considered only “partial” latencies as some EBV encoded genes are still expressed. Each type of latency has a distinct gene expression pattern. Latency III is the state of transformed lymphoblastoid B cells, and results in activation of a proliferation program in these cells. Six EBV nuclear antigen proteins, two latent membrane proteins (LMP), and EBV-encoded small RNAs (EBERs) are encoded during Latency III (4). In both Latency II and Latency I EBNA1 is expressed, and LMP1 and LMP2 are expressed in Latency II only. These phases are seen transiently or not at all during the progress of normal EBV infection in the healthy host (35). Latency II is typically observed in EBV related cancers or in germinal

center reactions which may be the transition of EBV infected B cells from Latency III to Latency 0 (36). “True” latency is only achieved at Latency 0, in resting memory-like B cells. No EBV encoded proteins are expressed in this phase of latency, and the EBV episome remains silent in the host nucleus (37). Cells in Latency 0 recirculate from the blood to the oropharynx, and comprise virtually all cells that can be detected in peripheral blood at both acute and latent stages of EBV infection (38). Only about one in a million circulating B cells is infected with EBV in healthy EBV infected adults (39). A complete cycle of EBV infection from acquisition to shedding may be seen in Figure 1-1.

The reactivation of EBV from latent to lytic infection *in vivo* is presumably important for viral transmission and for EBV related pathologies, but the triggers for reactivation remain ill-defined. Extrapolating from data obtained *in* vitro, it has been hypothesized that stimulation of the B cell receptor of infected cells may act as a trigger, or that certain types of other maturation signals may act as triggers (3).

Acquisition and Epidemiology

EBV infection is extremely common worldwide and approximately 90% of adults become antibody-positive before the age of 30 (40-42). A recent example is that 1037 (90%) of 1148 subjects 18 and 19 years old participating in the U.S. National Health and Nutrition Examination Surveys (NHANES) between 2003 and 2010 had IgG antibodies against EBV viral capsid (VCA) antigen, indicative of prior infection (1).

The prevalence of EBV antibodies in preadolescent children is lower, varying from 20% to 80% depending on age and geographic location. Factors clearly related to early acquisition of primary EBV infection include geographic region (reviewed in (43)), and race/ethnicity (1, 44). Other factors implicated are socioeconomic status (45-47), crowding or sharing a bedroom (45, 48), maternal education (49), day care attendance (47), and school catchment area (45).

Regarding race/ethnicity, it was recently shown that antibody prevalence across all age groups of U.S. children 6 to 19 years old enrolled in NHANES between 2003 and 2010 was substantially higher in non-Hispanic blacks and Mexican Americans than non-Hispanic whites (1). The greatest disparity in antibody prevalence was among the younger children, especially the 6- to 8-year-olds. Interestingly, the difference in antibody prevalence between whites and non-whites diminished during the teenage years. These NHANEs findings were confirmed (44) and extended to include younger children (18 months to 6 years of age) living in the Minneapolis-St. Paul metropolitan area. The Twin Cities study showed that the divergence in age-specific antibody prevalence between blacks and whites was clearly apparent by the age of 5 years.

Thus, family environment and/or social practices may differ among white and non-white families, which could account for this disparity in antibody prevalence in younger children. Alternatively, the explanation may be more closely linked to genetic differences between ethnicities, which is supported by the high frequency of concordance in same sex twins as opposed to non-twin siblings (50). Some racial groups have also been observed to shed higher levels

of virus. Non-white adults had significantly higher levels of EBV in oral wash samples than did white adults in a survey of parents with young children (Cederberg, et al., unpublished observations). This may or may not affect transmission among family groups. Within each race/ethnicity group, older age, lack of health insurance, and lower household education and income were statistically significantly associated with higher antibody prevalence.

Infectious mononucleosis occurs largely when EBV is first acquired after puberty. The age at which primary EBV infection is acquired may be increasing in developed countries (1, 51, 52). This is important to monitor because there is a complex interplay between age of acquisition, symptomatic versus asymptomatic primary infection, and the subsequent risk of EBV-associated cancers or autoimmune diseases. For example, younger age at the time of primary EBV infection among Kenyan infants was associated with elevated levels of EBV viremia throughout infancy, leading the investigators to postulate that these infants were at higher risk for endemic Burkitt lymphoma (53, 54). Another study found that Greenland Eskimo children acquired primary EBV infection at an earlier age and had higher titers of IgG antibody against VCA than did age-matched Danish children (55). The authors speculated that early infection with "a large inoculum of EBV" explained why Eskimos were at high risk for nasopharyngeal carcinoma versus Danes who were not. Nevertheless, late acquisition of primary EBV infection is also detrimental in several contexts. Adolescents and young adults are more likely to experience infectious mononucleosis during primary infection than children (56). Furthermore, multiple

sclerosis (MS) is an inflammatory autoimmune disease that may be linked to EBV infection and risk of MS is higher amongst individuals who have experienced infectious mononucleosis (57). Infectious mononucleosis also increases the risk of Hodgkin's lymphoma (58). Thus, since age of primary EBV infection is an important factor in infectious mononucleosis, it is an important consideration for EBV related diseases.

Infectious Mononucleosis

Of the diseases caused by EBV, infectious mononucleosis is the most well known. Infectious mononucleosis was the name coined by Sprunt and Evans (59) to describe a syndrome that resembled an acute infectious disease accompanied by atypical large peripheral blood lymphocytes. These atypical lymphocytes, also known as Downey cells (60), are actually activated CD8 T lymphocytes, most of which are responding to EBV-infected cells. EBV is medically important because of the severity and duration of possible acute illness and also because of its association with long-term consequences especially the development of certain cancers and autoimmune disorders (57, 61). From a basic biology standpoint, infectious mononucleosis also represents a natural example of initiation of a persistent viral infection.

Our prospective studies have determined that 75% of young adults between the ages of 18 and 22 develop typical infectious mononucleosis after primary EBV infection. Approximately 15% have atypical symptoms and 10% are completely asymptomatic (14; Balfour et al. unpublished observations). There are

two common presentations among symptomatic patients. The first is the abrupt onset of sore throat, which many patients say is the worst sore throat they have ever had. Patients may also notice a swollen neck that results from cervical lymph node enlargement. Parenthetically, anterior and posterior cervical nodes are usually equally enlarged. The second most common presentation is the gradual onset of malaise, myalgia ("body aches") and fatigue. Table 1-1 shows the frequency of signs and their median duration in 72 undergraduate students studied prospectively. Most signs/symptoms have a median duration of 10 days but fatigue and cervical lymphadenopathy persist for a median of 3 weeks. Other findings, seen in fewer than 20% of cases in our experience, include abdominal pain, hepatomegaly, splenomegaly, nausea, vomiting, palatal petechiae, periorbital and eyelid edema, and rash. Rash is seen more often in patients given penicillin derivatives, which is most likely due to transient penicillin hypersensitivity (62).

Feature	No. of Subjects (%)	Median Duration (Days)
Sore Throat	68 (94%)	10
Cervical Lymphadenopathy	58 (81%)	21
Fatigue	52 (72%)	20
Upper Respiratory Symptoms	46 (64%)	4.5
Headache	38 (53%)	9.5
Decreased Appetite	38 (53%)	9.5
Feels Feverish	34 (47%)	4
Myalgia (Body Aches)	33 (46%)	3

Table 1-1. Clinical features of primary EBV infections in 72 undergraduate students studied prospectively (48 women, 24 men; age range, 18-22 years).

Subclinical hepatitis documented by elevated levels of alanine aminotransferase occurs in approximately 75% of prospectively followed patients and, in some cases (5-10%), overt hepatitis develops with tender hepatomegaly and jaundice (Balfour et al. Unpublished observations).

Primary EBV infection in preadolescents has not been thoroughly investigated most likely because prospective studies in young children are logistically difficult to conduct. The assumption has been that the majority of primary EBV infections in children before puberty are asymptomatic but it is possible that symptoms are generally milder in children and thus overlooked clinically. Young children, especially those under the age of 4 years, may not develop a positive heterophile antibody response during primary EBV infection (63) and unless specific EBV assays are performed, the diagnosis will be missed.

It was previously thought that only 25%-50% of primary EBV infections in young adults result in symptomatic infection based on results from smaller prospective studies (15, 64-66). While our cohorts suggest that frequency is actually much higher, determining what factors differentiate symptomatic and asymptomatic seroconversion remains of great interest. Indeed, it has long been hypothesized that the magnitude of the CD8 T cell response may be the underlying cause of disease, specifically IFNγ produced by these cells which results in immunopathology. Heterologous immunity has been proposed as a potential mechanism of this phenomenon. Under this theory, memory cells primed by challenge with pathogens unrelated to EBV may be cross-reactive with EBV derived epitopes, thus enhancing the immune response to primary EBV

infection (67). Heterologous immunity may explain why infectious mononucleosis is more prevalent among adolescents than children (15). Cells specific for other common human viruses, however, only make up a small proportion of CD8 T cells expanded during infectious mononucleosis (68).

Likewise, viral load has also been hypothesized to affect both lymphocytosis and symptoms in both adolescents and children. Previous studies examining primary EBV infection in children have revealed that seroconversion often occurs with comparatively little CD8 T cell expansion (69, 70). Recent evidence from a cohort of Gambian infants showed that children may exhibit high viral loads consistent with those observed during infectious mononucleosis, but lack the typical CD8 T cell overexpansion, and are asymptomatic. While there was no lymphocytosis, a substantial EBV specific CD8 T cell response could be detected nonetheless, making up a surprising 16% of circulating CD8s (71). This finding suggests that disease progression related to acquisition of primary EBV infection is caused by an immunopathologic CD8 T cell response rather than direct virologic damage, although further study of this is warranted.

Complications of Acute Infectious Mononucleosis

Fortunately, serious complications during the acute phase of primary EBV infection are rare. Table 1-2 shows reported complications divided into those estimated to occur in at least 1% of patients and those that are seen in fewer than 1% of cases (72-76). Splenic rupture is the most feared complication, which has kept many athletes out of competition for weeks. Current consensus is that

athletes may return to contact sports 3 weeks after onset of infectious mononucleosis provided they are afebrile, their energy has returned to normal, and they have no other abnormalities associated with primary EBV infection (77).

Frequency of Complications	Complication
≥1%	Airway obstruction due to oropharyngeal inflammation Meningoencephalitis Hemolytic anemia Streptococcal pharyngitis Thrombocytopenia
<1%	Conjunctivitis Hemophagocytic syndrome Myocarditis Neurologic disorders (other than meningoencephalitis Neutropenia Pancreatitis Parotitis Pericarditis Pnemonitis Psychological disorders Splenic rupture

Table 1-2. Complications during acute primary EBV infection.

Although most symptoms associated with infectious mononucleosis resolve in a matter of months, there can be severe and lasting disease that develops following primary EBV infection. One of these complications may manifest in the form of chronic active EBV (CAEBV). Patients presenting with CAEBV generally exhibit signs that can occur during infectious mononucleosis such as fever, lymphadenopathy, splenomegaly and hepatitis, and show

markedly elevated levels of EBV DNA in the blood (78). Less frequently, patients may also present with lymphoma or hemophagocytic disease, a complication of EBV that is discussed in greater detail below (79). Interestingly, in many cases of CAEBV outside of the United States, particularly in Japan, EBV is reported to infect T or NK cells rather than its usual reservoir of B cells (78, 80). Several instances of B cell tropic CAEBV have also been reported, but these account for the minority of documented incidences (79, 81). While some patients have T or NK cell dysfunction, none of the subjects in a National Institutes of Health (NIH) study had mutations typically associated with EBV related immunodeficiencies and thus the disease observed in that study was considered to be largely idiopathic. The most successful treatment for CAEBV has been hematopoietic stem cell transplant. In the same NIH study, all but one of the patients who presented with CAEBV died within an average of six years unless a transplant was received. Those that survived all subsequently became negative for EBV DNA in the blood (82).

EBV may also cause hemophagocytic disease, which is alternately referred to in the literature as EBV-associated hemophagocytic syndrome or EBV-associated hemophagocytic lymphohistiocytosis (HLH). The relative rarity of any form of HLH stands as a barrier to diagnosis, and thus cases of EBV-HLH are even more uncommon. The disease is characterized by fever, splenomegaly and hematologic cytopenias, though the key laboratory signs are high levels of ferritin and soluble CD25 (83, 84). The distribution of EBV-HLH seems to be similar to CAEBV, focused mainly in Asian populations and infecting T or NK

cells in those groups (85) though B cells are also infected in other populations (86). EBV-HLH may be related to one of several primary immunodeficiencies discussed below, but EBV infection may be a triggering event even in the absence of an identified genetic condition. Interestingly, my thesis research showed that the peripheral blood gene expression signature observed during infectious mononucleosis highly resembles that of HLH (87). This reinforces links between primary EBV infection as a trigger in the initiation of HLH.

Diagnosis

Infectious mononucleosis due to EBV should be suspected in patients, especially teenagers and young adults, who present with an acute illness characterized by sore throat, cervical lymphadenopathy, fever and fatigue. Clinical signs that make the diagnosis more likely are exudative pharyngitis with swelling of the uvula and tonsils; periorbital and eyelid edema; and symmetrical cervical and postauricular lymphadenopathy.

A “heterophile test” using one of a number of commercially available antibody kits is most often done to support the clinical diagnosis (Table 1-3). Heterophile tests are relatively inexpensive and easy to perform. However, heterophile antibodies by definition are not specific. They are IgM class antibodies directed against mammalian erythrocytes. False positive heterophile tests have been reported in myriad conditions including other acute infections, autoimmune disease, and cancer (88-92). Although heterophile tests are most commonly used to diagnose infectious mononucleosis, the U.S. Centers for

Disease Control and Prevention has recently advised against them "for general use" because of their non-specificity and the possibility of false negative results especially in young children (93). In our experience, however, if the clinical picture is typical of infectious mononucleosis and the heterophile antibody test is positive, no additional diagnostic procedures are necessary.

Stage of Infection	VCA IgM	VCA IgG	EBNA-1 IgG
Naïve	Negative	Negative	Negative
Acute Primary[a]	1—2+	Neg—1+	Negative
Subacute[b]	3—4+	2—4+	Negative—1+
Convalescent[c]	Negative—3+	3—4+	Negative—2+
Past[d] (90—95% of cases)	Negative	3+	3—4+
Past[d] (5—10% of cases)	Negative	3+	Negative

[a] 0 to 3 weeks after onset of illness
[b] 3 weeks to 3 months after onset of illness
[c] 3 to 6 months after onset of illness
[d] > 6 months after onset of illness

Table 1-3. Staging EBV Infection by enzyme immunoassay patterns.

No single antibody test is perfect for confirming the diagnosis of primary EBV infection. Most patients (75%) will have VCA IgM antibodies at the onset of clinical illness and 95% eventually make them (Table 1-3). In prospective studies of EBV-naïve college students, we detected VCA IgM by EIA as early as 8 days before onset of symptoms. The median first day of detection was 2 days after onset of illness (Figure 1-2). However, a problem with IgM class antibody tests in general is cross-reactivity with related pathogens. In the case of VCA IgM antibodies, false positive results have been reported especially with cytomegalovirus infections (94).

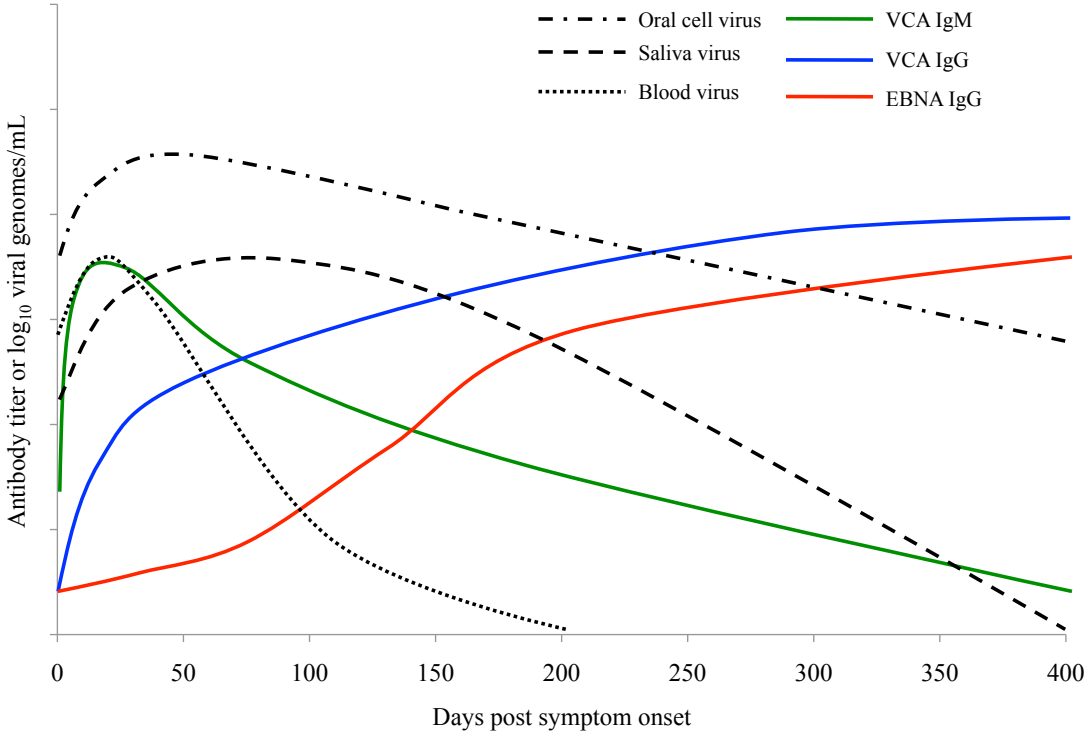


Figure 1-2. Kinetics of EBV viral load and antibody responses in subjects with primary EBV infection. Depicted are viral loads in whole blood, saliva, and oral cell pellets (black lines) as well as IgM and IgG antibodies to VCA and IgG to EBNA1 (colored lines). Note: the limit of detection of the EBV viral genomes in blood was 200 copies per mL of whole blood.

Depending on the assay platform and antigen used in the assay, VCA IgG antibodies are first detected during the first month of illness. IgG class antibody against the p18 component of VCA develops later than does IgG against the p23 component (see Figure 1-3). Using an EIA with p18 as the antigen, we found in prospective studies that the median first day of detection after onset of illness was 31 with a very wide range of 1—118 days. Nearly everyone who experiences a primary EBV infection develops IgG antibodies to VCA (14), so this is the best single test to verify a previous EBV infection. It is superior to

EBNA-1 antibody tests because antibodies to EBNA-1 are slow to develop and 5—10% of patients never make a response. Trends in viral load and antibody titer are shown in figures 1-2 and 1-3.

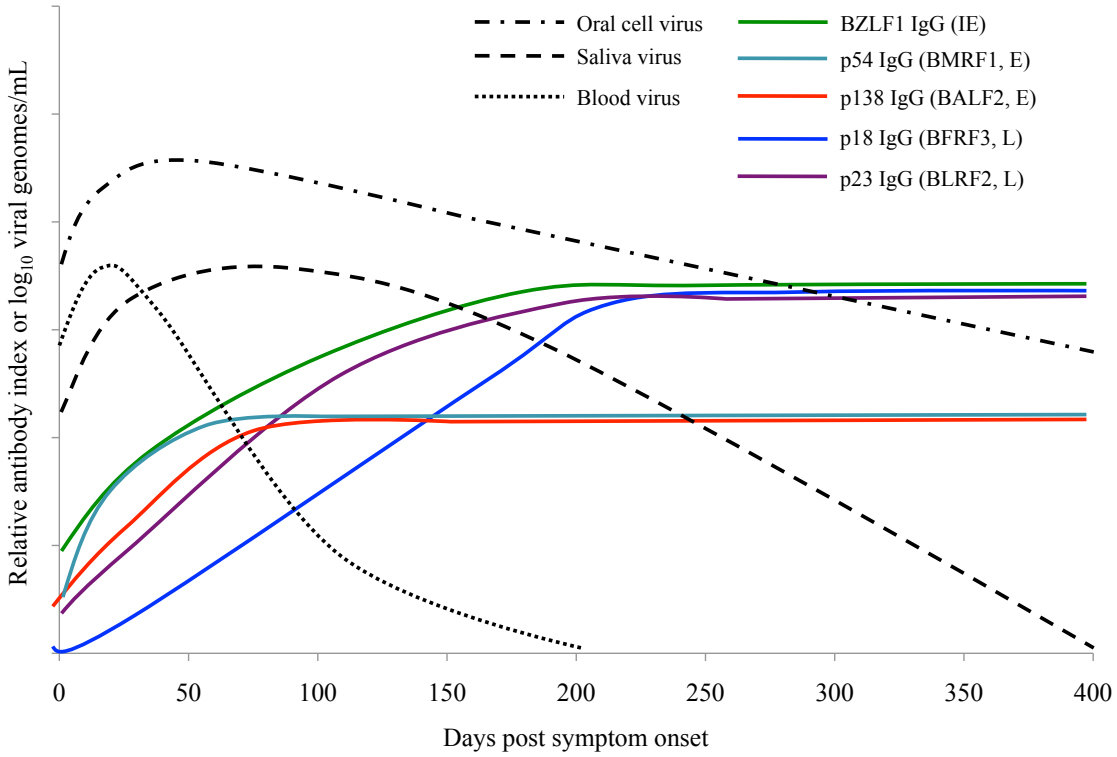


Figure 1-3. Kinetics of antibody responses to additional EBV antigens as determined by immunoblot. Depicted are viral loads in whole blood, saliva, and oral cell pellets (black lines) as well as the relative kinetics of antibodies generated against the EBV antigens p18, p23, BZLF1, p138, and p54 (colored lines). The gene name of each antigen and the stage of expression (IE: immediate early, E: early, or L: late) are indicated in parentheses.

Antibodies against EBNA-1 are slow to appear with a median first day of detection of 91 days (Figure 1-2). Because of this, the presence of EBNA-1 antibodies during an acute illness rules out primary EBV infection. In general, the vast majority of EBV infections in immunocompetent patients can be staged by

assaying a blood sample for VCA IgM, VCA IgG and EBNA-1 antibodies. Western blots or immunoblots can be used to confirm results of screening tests and also stage EBV infections (95, 96).

Measurement of IgG antibodies against EBV early antigen (EA) are not useful for the diagnosis of primary infection because only 60—80% of acutely ill patients are positive and EA antibodies can be found for years in 20% of healthy individuals (93, 97). When the available antibody data do not distinguish the stage of an EBV infection, IgG avidity assays may be useful (95, 98). The principle is that during the course of infection, antibodies with high binding strength to their target are selected. In other words, IgG antibodies during the acute phase of infection do not bind to their target as tightly as antibodies produced during convalescence. Low avidity antibodies can be dissociated from their target by exposure to urea or another chaotropic reagent. Avidity is determined by comparing the amount of antibody detected with and without urea treatment.

The best test for diagnosing and monitoring EBV infections in the immunocompromised host is the blood viral load (or quantitative EBV DNAemia assay) usually performed on a PCR platform (99). Most of these infections are not primary but there are a few that are and result in classic infectious mononucleosis. The monitoring concept is to anticipate the risk of impending EBV disease based on sequential changes in blood viral load. A threshold amount, which varies from center to center, is established that triggers intervention with changes in immunosuppressive and/or antiviral drug regimens.

Genetic Susceptibility

Given the disparities in antibody prevalence among populations of different racial backgrounds observed in surveys of children in the United States, there may exist variances in the genetic susceptibility of certain race/ethnicities to EBV. This theory is supported by the incidence of infectious mononucleosis in family groups. A study examining infectious mononucleosis concordance in twins from the California Twin Program found monozygotic twins were twice as likely to both develop infectious mononucleosis than were dizygotic twin pairs. When the analysis was limited to same sex dizygotic twins, the risk in those groups was higher (100). These findings were then further expanded to include first-degree relatives in a large study of Danish families surveyed by governmental registries. The rate ratios for same sex twins were highest, followed by groups of siblings (50). It is important to note that siblings tend to have similar environments and behaviors, which may explain why the degree of concordance is so high.

Further evidence lends credence to a genetic basis for susceptibility to infectious mononucleosis. Recently, the HLA locus was identified as a major factor influencing antibodies to EBNA-1 in large Mexican American families (101). Although the authors interpreted this to be a genetic influence on susceptibility to EBV infection, not all individuals who are infected with EBV make a strong antibody response to EBNA-1. Therefore it is possible that the HLA locus influences the response to infection rather than the infection rate per se. In regard to infectious mononucleosis, the effect on the adaptive response to

primary infection may be sufficient to influence whether or not primary infection is symptomatic. Class II MHC is also required for viral entry into the cell, although whether or not there are alterations in viral entry efficiency between HLAs prevalent in different racial groups has not been explored.

Immune Responses to EBV

The viral dynamics and EBV-specific antibody responses during primary EBV infection are illustrated in Figures 1-2 and 1-3. High viral loads in both the oral cavity and blood are detected around the time of symptom onset in infectious mononucleosis and accompanied by production of IgM antibodies to EBV VCA and an extraordinary expansion of CD8 T lymphocytes (14). Of particular interest is the response of cytotoxic CD8 T cells, which have been shown to be important in the control of EBV infected B cells as evidenced by the disease that occurs in patients who lack elements of CD8 T cell function such as the ability to interact with and kill infected B cells (102, 103).

During infectious mononucleosis when there are very high numbers of circulating CD8 T cells, many of these cells are EBV specific and directed toward lytically expressed proteins from the immediate early and early stages of the lytic cycle with particular predilection for the immediate early. Cells specific for some late antigens tend to emerge only after the patient has been infected for some time as discovered by generating T cell clones from infectious mononucleosis patients (104). The case of latency, however, is rather different and is often dependent on the relevant HLA type of the EBV infected individual in question.

Immunodominant epitopes for the most prevalent HLA types generally include those derived from latently expressed proteins, especially EBNA-2 and EBNA-3, although some patients develop a strong population of CD8 T cells specific for less readily expressed antigens such as EBNA-1 (105, 106).

Both CD8 and CD4 T cells require cell-to-cell contact in order to become activated and perform related functions (107, 108). Although total CD4 numbers do not increase appreciably during infectious mononucleosis, evidence exists to support the concept that CD4 T cells are activated and help control infected B cells. Using major histocompatability complex (MHC) II tetramers, it was shown that several lytic antigens are recognized by CD4 T cells during acute infection and that these cells are maintained at low levels in the blood of EBV infected hosts (109). That study also revealed that the response to different antigens varies: CD4(+) T cell responses to EBNA1 did not develop until much later, which likely explains the delay in EBNA1 IgG antibody responses.

Natural killer (NK) cells are also emerging as important players during infectious mononucleosis. Several immunodeficiences involving T and NK cells and/or their cytolysis pathways result in severe EBV related outcomes (110, 111). These include familial hemophagocytic lymphohistiocytosis 2 (FHL2), X-linked lymphoproliferative syndrome (XLP), XIAP deficiency, and X-linked immunodeficiency with Mg+2 defect (XMEN) disorders, and are discussed in detail later in this chapter.

The value of NK cells specifically, was suggested by the observation that NK cells preferentially killed EBV infected cells as the virus entered the lytic cycle

(112). The role of NK cells *in vivo* was investigated using a humanized mouse model, where NOD-scid γc-/- (NSG) mice were reconstituted with CD34+ lin- hematopoietic stem cells (113, 114) and infected with the B95.8 strain of EBV and monitored for signs of infectious mononucleosis such as CD8 lymphocytosis and viremia. Animals depleted of NK cells experienced more severe EBV related disease (115). It is interesting to note that NK depletion after an established EBV infection did not have significant effects, in contrast to the effect of depletion before infection. Given the gap in the robustness of responses observed between tonsillar and peripheral blood NK cells, it is possible that peripheral NK cells during the systemic infectious mononucleosis phase are less important than those during early infection in the oropharynx (116). Indeed, studies in humans have disagreed about the state of NK cells in peripheral blood during infectious mononucleosis. Work from Williams et al. showed an inverse correlation between NK cell numbers in the periphery and virus in the blood (117). In contrast, a larger prospective study found a positive correlation (14). Thus, the interplay between NK cells and blood virus in human subjects needs further study. Of course, the specific type or subset of NK cells may be more important than total numbers. NKG2C+ NK cells have been shown to specifically respond to and play a crucial role in immunity to cytomegalovirus (118). However, NKG2C+ NK cells do not expand upon EBV infection (119). Rather NK cells expressing NKG2A and CD54 could be found in higher numbers in the tonsils of EBV carriers (120) and in the peripheral blood during acute infection (119). In a study of pediatric infectious mononucleosis patients, it was recently shown that $CD56^{dim}$ NKG2A+

KIR- NK cells preferentially proliferate in response to EBV infected cells during acute infection (121).

EBV also seems to have evolved mechanisms to interfere with the activation of NK cells during viral replication. The protein encoded by the EBV open reading frame BILF1 downregulates expression of HLA-A and HLA-B on the surface of infected cells but not HLA-C, which is inhibitory to NK cells (122). Several specific populations of NK cells have been implicated in limiting the transformation of B cells by EBV in vitro. When exposed to dendritic cells (DCs) prepared with EBV, $CD56^{bright}$ CD16- NK cells were preferentially primed and were able to limit B cell transformation in vitro in an interferon (IFN) γ dependent manner. Interestingly, tonsillar NK cells were much more efficient than NK cells derived from peripheral blood (116, 123). Further understanding of NK cell recognition of EBV infected cells and their responses during human infection are needed at this point.

Despite our growing understanding of the innate and adaptive immune response to EBV, it remains unclear why primary EBV infection leads to infectious mononucleosis in adolescents yet is most often asymptomatic in young children. It is possible that adolescents receive a larger initial virus inoculum when transmission occurs through deep kissing. Our studies did not reveal any correlation between virus copy number in the oral cavity and severity of illness (14), however peak virus copy number in the oral cavity may not directly reflect the initial virus inoculum, nor is it possible to measure the initial virus inoculum with natural infection. Another idea put forward is that infectious mononucleosis

in adolescents may reflect the response of cross-reactive memory CD8 T cells. For example influenza specific CD8+ T cells might cross-react with EBV (124) and as adolescents are presumably more likely to have high numbers of influenza specific CD8 T cells, they could react more strongly with EBV. However, we have not seen evidence of influenza-EBV dual specific CD8 T cells in our cohort (68) and it remains debatable whether pre-existing (cross-reactive) CD8 T cell immunity to EBV would increase or decrease in severity of infectious mononucleosis. A CD8 T cell peptide epitope vaccine was effective in generating EBV specific CD8 T cell responses, and there was no incidence of infectious mononucleosis in the vaccinated group, although the study was small (125). An exciting proposition has arisen from recent data that implicate NKG2A+ NK cell as important in EBV control (119, 121). Azzi and colleagues showed that $CD56^{dim}$ NKG2A+ KIR- NK cells were found at significantly lower levels in the peripheral blood of adolescents and adults compared to children, suggesting that decreased NK mediated immune control of EBV could explain why adolescents and adults experience infectious mononucleosis more frequently than do children.

During the convalescent period of infectious mononucleosis (3 to 6 months postinfection), the number of CD8+ T cells declines to normal levels (14). Previous work in mice suggested that infection with hepesviruses may cause long term changes to the “readiness” of host immune cells, thus priming them for subsequent bacterial infections (126). These effects were later shown to be transient in that model (127), but work in human subjects showed that there were no long-term gene expression changes observable in peripheral blood

mononuclear cells following acquisition of EBV (87). The virus is maintained in resting memory-like B cells. Probably the most interesting phenomenon that occurs during this phase of infection is related to the levels of antibody that are produced and maintained by latently infected hosts.

Some peculiar trends exist in the antibody response to EBV. For example, antibodies against EBNA-1 display an unusually long delay between virus acquisition and the presence of anti-EBNA-1 IgG, as it generally appears only after a patient with infectious mononucleosis has convalesced (128, 129). This is especially odd given the high levels of class switched antibody toward EBV antigens that can be measured in many patients presenting with infectious mononucleosis, including responses to latently expressed gene products of EBNA-2 and EBNA-3 (109) (Figures 1-2 and 1-3). The delayed kinetic might be associated with alanine-glycine rich regions within the structure of EBNA-1's protein product, which has been shown to inhibit proteosomal processing of relevant epitopes (130). That this is the case, however, is not clear and may have to do more with accumulation of protein released from cells for cross presentation. In contrast, high levels of antibody specific for the immediate early antigen BZLF1 are maintained for the life of the host. This is likely because BZLF1 is expressed by cells undergoing viral reactivation and thus is more frequently presented to B cells in latently infected hosts in comparison to proteins that are expressed later in the viral replication process (131).

Primary Immunodeficiencies

X-linked lymphoproliferative syndrome (XLP) is a disease characterized by anemia, hypergammaglobulinemia and lymphohistiocytosis. Generally the boys that present with this disease exhibit massive cellular responses to primary EBV infection that results in hemophagocytic pathology, even though they are simultaneously unable to control EBV transformed B cells (132). It was then discovered that the main deficiency involved with XLP is in the SLAM family member slam associated protein (SAP), which is encoded by the human gene *SH2D1A*. Mutations in this gene disrupt the ability of T cells and NK cells to interact with B cells, resulting in a lack of immunoglobulin class switching and meaning T cells and NK cells cannot recognize B cell targets to induce death (133, 134). A study of XLP patients showed through random X chromosome inactivation that only CD8 T cells that have a functioning copy of *SH2D1A* in mothers of boys with XLP are able to kill EBV infected B cells (102) and that boys with CD8 T cells that spontaneously recover SAP expression have much better outcomes (135). A similar disease exists in the form of XIAP deficiency, which causes a defect in the function of caspase activity, and thus cell mediated cytotoxicity. Patients who lack functional XIAP also have difficulty controlling EBV infected cells, because they also cannot induce lysis of EBV infected targets (132).

The Fcγ receptor CD16 is necessary for proper NK cell function by allowed for cytolysis of target cells in an antibody dependent manner. It was first recognized that some patients with recurrent infections, including herpesviruses, had a mutation in the gene encoding CD16 (136, 137). Later it was discovered

that the specific homozygous missense mutation involved caused CD16 not to associate with its signaling partner CD2 on the cell surface. Thus, signaling through CD16 to induce spontaneous NK cell cytoxicity was eliminated even after engagement of CD2 (138).

Another X-linked syndrome observed in boys involves the magnesium transporter MAGT1 and is known as X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia (XMEN). Patients affected presented with defects in T cell activation as well as high EBV viral loads and over time developed EBV related disease (139). Sequencing of the X chromosome in two brothers revealed that both had a deletion in the region of *MAGT1*. Impaired magnesium transport resulted in reduced signaling following antigen receptor engagement, particularly interfering with calcium flux which is necessary during T cell activation (140). Investigators then isolated cells from several patients with similar symptoms and supplemented their media with magnesium, thereby restoring much of the NK and T cell function to these cells. Results were later extrapolated to dietary supplements in XMEN patients, and helped abrogate related symptoms (141).

Serine threonine kinase 4 (STK4) is required for the activation of the forkhead box proteins FOXO1 and FOXO3. Both of these transcription factors have important roles in T cell homing, function, and homeostasis, especially during persistent viral infections (142-145). Without functional STK4, these patients experience recurrent episodes of herpesvirus reactivation, in particular those with cutaneous manifestations such as HSV and VZV. Approximately half

of patients presenting with STK4 mutations also have difficulty controlling EBV and have EBV related B cell pathologies. Successful treatments for STK4 deficiency have included anti-CD20 and hematopoietic stem cell transplant (146). Another protein known to affect the response to primary EBV infection is the TNF receptor family member CD27. Patients from three independent families presenting with severe primary EBV infection or EBV related lymphoproliferative disease were found to have a homozygous mutations in the gene encoding CD27. It was initially thought that the role of CD27 must be related to its expression in regard to the development of T or B memory phenotype B cells, but this was found not to be the case (147, 148). What exactly drives disease in these instances is not well understood.

EBV Associated Diseases

EBV has been shown to be the causative agent of about one percent of the worldwide human cancer burden. In particular, EBV infection is associated with neoplasia of lymphoid origins including endemic Burkitt's lymphoma (eBL) and Hodgkin's lymphoma (HL), and epithelial origins including nasopharygeal carcinoma and gastric carcinoma. EBV is considered the etiologic agent in 95% of cases of eBL, which occur in regions where malaria is common (149). Likewise, EBV can be detected in a high proportion of HL cases in underdeveloped nations, but accounts for less than half of cases in Western countries (150). It is important to note that incidence of infectious mononucleosis is exceptionally low in Southeast Asia and equatorial Africa where EBV infection

during childhood is nearly ubiquitous, thus it might be extrapolated that infectious mononucleosis does not have a strong correlation with either eBL or HL in these areas (151). Emerging evidence suggests that previous presentation with infectious mononucleosis can increase the risk of HL (58). While associations between infectious mononucleosis and epithelial carcinomas have not been explored, the presence of EBV in tumors from nasopharyngeal and gastric carcinoma patients is well documented. About 10% of human gastric carcinomas are EBV positive (152). Like eBL and HL seen in underdeveloped nations, nasopharyngeal carcinomas from endemic regions are virtually all positive for EBV DNA (153), with these tumors thought to be derived from a single EBV infected epithelial cell (154, 155).

It is possible that achieving a very high viral titer in the blood at any point in life predisposes individuals to subsequent EBV related cancers. For example, patients who present with eBL and endemic nasopharygeal carcinomas live in malaria endemic areas. Evidence shows that patients being treated for malarial disease can have extremely high titers of EBV in the blood (156). Titers of this magnitude are seen exclusively in patients presenting with infectious mononucleosis in developed countries. Thus, it may be possible to reduce occurrences of cancer with prophylactic or therapeutic vaccines aimed at preventing primary EBV infection or at the very least reducing the set point at which the virus is maintained in these individuals.

In recent years, infectious diseases have been emerging as possible triggers for autoimmune disorders. EBV infection in particular has come to be

highly associated with occurrence of MS. EBV as a causation factor in MS was first proposed over thirty years ago (157). Many correlative observations for this trend exist, including a low incidence of infectious mononucleosis and MS in developing countries, and MS usually first manifests after the adolescent years during which EBV would be acquired, increasing at a rate of 11% per year following primary EBV (57). There is also a high association between patients who recall having infectious mononucleosis and subsequent development of MS (158-160). Furthermore, MS in EBV negative individuals occurs very infrequently (41, 161).

A causative role for EBV was supported by examination of the antibody profiles of patients with MS, scrutinizing the viral loads, epitope specificity, and quantity of antibodies, especially those against EBNA. The risk of MS increases positively with levels of circulating anti-EBNA antibodies (162-165). The ability to discriminate MS cases and controls was substantially enhanced by the inclusion of quantitative measures of the anti-EBNA-1 response to EBV infection (166). Higher loads of EBV DNA can also be found in the cerebrospinal fluid of patients with relapsing remitting MS as compared with controls (167). Interestingly, the strong genetic association of MS with particular human leukocyte antigen (HLA) alleles primarily reflects the association with anti-EBV responses (101). Researchers recently treated an MS patient with autologous T cells expanded by exposure to EBV antigens. Transfer of the EBV specific CD8 T cells resulted in a decrease in anti-EBV antibody titres as well as in the size and number of MS related lesions in the brain (168).

Animal Models

One of the major barriers to studying many human viruses is the lack of a small animal model. EBV belongs in this group because it only infects primates. Although studies have been performed with murine gammaherpesvirus-68 (MHV-68) there are important genetic differences between that virus and EBV. In order to directly evaluate the effects of EBV on various lymphoid compartments in vivo, efforts toward developing feasible methods for modeling human infections have resulted in the creation of humanized mice. Humanized mice have only become a viable option for studying human diseases within the last ten years or so due to low engraftment of human cells even in animals with severe combined immunodeficiency (SCID) or knockout of one of the recombinase activating genes (RAG). With the advent of common gamma chain (a receptor component for IL-2, 4, 7, 9, 15, and 21) knockouts and appreciation of non-obese diabetic mouse polymorphisms that influence phagocytosis, engraftment improved dramatically.

The two types of mice most commonly employed are the previously mentioned NSG and the BALB/c RAG-/- γc-/-. Cells derived from either human fetal liver, human fetal thymus, or CD34+ hematopoietic stem cells are then transferred into neonatal mice to reconstitute their immune systems (169). Of these two mouse strains, the NSG mouse has more complete reconstitution of the T and NK compartments and could be maintained for 22 weeks with latent virus detectable and without developing tumors or other EBV related pathology

(170). This model has also been shown to give an approximation of human immune components (113, 114). Mice are bred onto a transgenic HLA background. This then allows for thymic selection of the human derived cells and later specific responses during infection of these animals with EBV (171, 172).
It is important to note, however, that study of EBV within this context may neglect important aspects of interplay between EBV and epithelial cells, which have been shown to be important during the replication of EBV, chiefly within the oropharynx (23). Nevertheless, humanized mice represent a significant step forward to investigating cellular responses in vivo.

In particular, the roles of certain subsets of cells have been interrogated through the depletion of these populations prior to infection with EBV. A significant gap in our understanding of the early innate response to EBV existed and had only been addressed in vitro prior to the advent of this model. Using the NSG mouse and virus obtained from the B95-8 cell line, investigators can now effectively emulate events that occur early during infection in the peripheral blood. Humanized mice exhibit classical features of infectious mononucleosis such as elevated CD8 T cell counts and high levels of IFNγ (115). Specific deletion of subsets of NK cells could then be performed to examine which were most important during the response to primary EBV infection. Furthermore, the roles of adaptive immune cells may be examined as well. Investigators looking at CD4 and CD8 T cells found EBV specific T cells were HLA restricted and responded to autologous lymphoblastioid cell lines. When either CD4 or CD8 T cells were depleted from these mice, the mice developed EBV related pathologies (170).

These results are not particularly surprising given the same pattern can be observed in primary human immunodeficiencies as previously described, but they provided important evidence that the humanized mouse can be an appropriate tool for asking questions about the response to EBV. Mouse models, however, neglect nearly all aspects of initial infection as events in the oral cavity cannot occur as EBV does not have tropism for murine epithelial cells. In order to examine infection of the tonsil and oral epithelium, primate models might be preferred as there is established infection in the oral cavity similar to EBV in humans.

Studies from Japan have suggested that EBV infection may be modeled in rabbits (173, 174). Animals in these studies were alternately inoculated intravenously, intranasally, and perorally with EBV derived from the B95-8 cell line. Most animals had only transient levels of virus detectable in the blood, but two had consistent viral titers. Both T and B cells appeared to be infected in these incidences (173, 174). It is important to note that while early antigen IgG titers were maintained, VCA IgG antibodies were transient even when very high quantities of virus were used (174). Though not a model of infection with a great deal of similarity to infectious mononucleosis, rabbits may still provide interesting insight with regard to the kinetics and magnitude of antibody responses to EBV, which has implications for the development and testing of humoral component vaccines such as the gp350 subunit vaccine.

Non-human primates are the other major option for investigating EBV infection in vivo. The gamma herpes virus LCV exists in two types: that which

infects old world and that which infects new world primates. The LCV that infects old world primates has higher genetic similarity to EBV than new world LCV. The open reading frames of rhesus LCV have 28-98 % amino acid identity with EBV (175). Thus, rhesus LCV is used to model EBV in rhesus macaques. The symptoms of EBV and LCV infection are very similar, and LCV can be given orally to emulate natural infection routes in humans. One difference is that the incubation period of LCV is generally shorter, lasting about three weeks rather than six.

LCV can be manipulated using a bacterial artificial chromosome system to allow for mutation of the LCV genome. This enables researchers to examine the possible effects of EBV homologues in vivo. For example, BARF-1 has been shown to bind and inhibit the signaling of colony stimulating factor 1 (176, 177), which can promote the maturation and maintenance of type I interferon producing plasmacytoid DCs (178). Knocking out BARF-1 in LCV resulted in more favorable outcomes and lower viral loads in infected rhesus macaques (179).

The LCV model can also be used to test potential prophylactic or therapeutic vaccines. An LCV gp350 subunit vaccine protected against infection and reduced the viral set point in rhesus macaques (180). More recently, Leskowitz and colleagues showed that an adenovirus based vaccine encoding LCV EBNA-1 induced expansion of CD4+ and CD8+ T cells specific for EBNA-1 in rhesus macaques with natural persistent LCV infection (181).

Therapeutics and Prophylactics

Given the disease burden associated with acute and chronic EBV diseases, development of an EBV vaccine has long been a priority for researchers in the field. The National Cancer Institute recommended that more clinical trials be conducted to test the safety and efficacy of a vaccine to prevent infectious mononucleosis and cancers caused by EBV (61). Although the first phase 1 trial for a prophylactic EBV vaccine occurred almost twenty years ago (182), there has been relatively little progress since. In total, three prophylactic vaccines have been tested in humans, and although all proved at least moderately immunogenic, none provided sterilizing immunity (183). However, sterilizing immunity is probably not necessary to impact symptomatic disease caused by primary EBV infection. For example, a phase 2 trial in Belgium showed that vaccination with a gp350 subunit adjuvanted vaccine reduced the number of cases of infectious mononucleosis (184).

Whether or not a vaccine exclusively targeting gp350 is sufficient to prevent EBV related disease, however, is unknown. In the case of epithelial neoplasia, it seems less convincing on the grounds that gp350 is not strictly required for viral entry into epithelial cells and can be achieved via the viral proteins gH and gL, albeit less efficiently (30). However, it is possible that preventing B cell infection in vivo could impact epithelial infection, given EBV's interesting switch tropism. Increasing the range of the vaccine to include other proteins necessary for epithelial entry such as the aforementioned gH or gL,

might improve the efficacy of a vaccine with the goal of preventing EBV positive lymphomas and carcinomas.

Treatment

There is no currently accepted specific treatment for infectious mononucleosis. While it is clear that acyclovir and valacyclovir and have an antiviral effect in vivo, a clinical benefit has not been convincingly demonstrated to date (185). Ganciclovir and valganciclovir have been used treat EBV infections in immunocompromised hosts but there are no controlled trials demonstrating clinical efficacy. Corticosteroids are often prescribed to treat inflammatory complications such as airway obstruction or autoimmune phenomena such as anemia and thrombocytopenia, but the value of these drugs is controversial and they may impair clearance of the viral load (186).

Thesis Statement

EBV is one of the most important human pathogens. Although this virus was discovered more than 50 years ago and infects more than 90% of the worldwide population, there are large gaps in our knowledge of its epidemiology and pathogenesis. Our future challenge is to focus research on the following five gaps.

1. We don't know how EBV is transmitted to young children.

2. We don't know why some adolescents and young adults become very ill from a primary EBV infection while others remain completely asymptomatic.

3. We don't have an approved specific treatment for EBV infections.

4. We don't have an approved EBV vaccine.

5. Finally, we don't know the mechanism by which EBV induces malignancies or autoimmune diseases. In terms of EBV-associated cancer, we do know a reasonable amount about how this virus infects and transforms lymphocytes and epithelial cells. What we don't understand is how these cells escape immune recognition.

This thesis attempts to address these issues by using systems biology and cellular phenotyping to examine both the incubation period and acute symptomatic phases of EBV related infectious mononucleosis. Overall, we have found that there are several previously uncharacterized virological and immunological events that occur prior to symptom onset. First, EBV disseminates from the oral cavity to the periphery much earlier than previously thought, presumably in latently infected “Latency 0” B cells. Second, a type I IFN signature occurs most robustly before symptom onset and occurs concurrently with dissemination of virus to the periphery. Third, plasmacytoid dendritic cells are significantly diminished in numbers and frequency in the circulation up to ten days before symptom onset and do not recover for a number of weeks.

Chapter 2

Primary EBV infection induces an expression profile distinct from other viruses but similar to hemophagocytic syndromes

Abstract

Epstein-Barr Virus (EBV) causes infectious mononucleosis and establishes lifelong infection associated with cancer and autoimmune disease. To better understand immunity to EBV, we performed a prospective study of natural infection in healthy humans. Transcriptome analysis defined a striking and reproducible expression profile during acute infection but no lasting gene changes were apparent during latent infection. Comparing the EBV response profile to multiple other acute viral infections, including influenza A (influenza), respiratory syncytial virus (RSV), human rhinovirus (HRV), attenuated yellow fever virus (YFV), and Dengue fever virus (DENV), revealed similarity only to DENV. The signature shared by EBV and DENV was also present in patients with hemophagocytic syndromes, suggesting these two viruses cause uncontrolled inflammatory responses. Interestingly, while EBV induced a strong type I interferon response, a subset of interferon induced genes, including *MX1, HERC5,* and *OAS1*, were not upregulated, suggesting a mechanism by which viral antagonism of immunity results in a profound inflammatory response. These data provide an important first description of the response to a natural herpesvirus infection in humans.

Introduction

Epstein-Barr virus (EBV) is a herpesvirus that causes lifelong infection in humans. EBV is estimated to be a causative agent in 1% of all human cancers, (187) including various lymphomas, and nasopharyngeal and gastric carcinomas;

(188) and to contribute to autoimmune diseases such as systemic lupus erythematosus (SLE) (189) and multiple sclerosis. (190) Nonetheless, the most common consequence of EBV infection is infectious mononucleosis (IM). (35) Primary acquisition of the virus by children before puberty does not generally cause recognizable symptoms, but those who become infected during or after adolescence have a high likelihood of developing IM. EBV infection is trending older, particularly in developed countries, (51) and IM represents a growing health issue. There is currently no vaccine and no effective treatment for EBV infection. IM is distinct from acute infection with other viruses in that it is characterized by a lengthy incubation period and severe lymphocytosis. The incubation period for EBV is about six weeks in length, (13) contrasting starkly with symptomatic viral illnesses such as influenza where the incubation period is only a few days. In addition, the lymphocytosis observed in IM reflects a profound activation and expansion of EBV specific, and to a lesser extent, bystander CD8+ T cells. (68, 106)

In order to gain insight into EBV pathogenesis, we examined the host response to EBV using a genomic approach. Systems biology is emerging as an important tool for understanding the human response to infection that can provide novel means of diagnosis and insight into disease mechanisms. (191) Recent studies have reported genomic changes occurring in B cells infected with EBV *in vitro* (192) or EBV+ Burkitt Lymphoma cell lines. (193) However, there is a dearth of understanding in current research about the host gene changes that occur during natural viral infection with EBV in otherwise healthy humans and

whether or not these infections lead to long lasting changes in the immune system. We sought to address these issues by performing transcriptomic studies on human subjects participating in a prospective study of primary EBV infection acquired naturally. We then compared these data with gene expression analyses of other acute viral infections, systemic illnesses, and interferon (IFN) driven immune responses. We found the human immune response to EBV has a distinct and reproducible gene expression signature that more closely resembles the dysregulated innate immune responses observed during cytokine storm illness than what is seen during other acute viral infections. Furthermore, we identified a subset of genes that represent likely targets of viral immune evasion mechanisms.

Results

Prospective analysis of EBV infection in young adults

EBV naïve subjects were recruited from freshman classes at the University of Minnesota during 2006 and 2007. Enrolled study subjects were screened for exposure to EBV on an ongoing basis by testing blood or throat wash samples taken every four to eight weeks. Samples were evaluated for EBV genomes by qPCR and/or antibodies to viral antigens. Of 143 subjects followed in the study, 66 experienced primary EBV infection during their undergraduate years. (194)

Due to the long incubation period, the exact date of viral acquisition is not known. Instead, date of symptom onset was used for timing, since this date was

well defined for each subject. For acute microarray analysis, we chose eight subjects with peripheral blood samples taken near the onset of symptoms, and compared them to samples taken at least three months before infection. These eight subjects plus an additional two were chosen for analysis of latency, with latent samples being defined as at least >200 days after infection (Table 2-1). Baseline and latency samples were chosen from timepoints when the subjects did not report symptoms of any other illness and had not reported illness for at least two weeks prior. Further analysis was performed by PCR on an additional 44 subjects following initial evaluation of these eight by microarray as described below.

Patient ID			PRE		ACUTE		LATENT
5027		Day*	-137		-15		566
SOI	5	VCA IgM#	(-)		-		
Gender	Female	VCA IgG#	-		-		+++
Duration	42 days	Oral virus**	(-)		800		11400
CMV	neg	Blood virus**	(-)		-		-
5088		Day	-118		-4		406
SOI	4	VCA IgM	(-)		-		
Gender	Male	VCA IgG	-		-		+++
Duration	21	Oral Virus	(-)		15,000		-
CMV	neg	Blood virus	(-)		-		-
5509		Day	-119		-4		563
SOI	3	VCA IgM	(-)		-		
Gender	Female	VCA IgG	-		-		(+++)
Duration	8 days	Oral Virus	(-)		42,500		-
CMV	neg	Blood virus	(-)		18,800		-
5342		Day	-115		0		704
SOI	4	VCA IgM	(-)		+ / -		
Gender	Female	VCA IgG	-		-		+++
Duration	27 days	Oral Virus	(-)		67,000		1300
CMV	neg	Blood virus	(-)		5,000		-
5483		Day	-97		3		231
SOI	3	VCA IgM	(-)		+++		
Gender	Female	VCA IgG	-		-		+++
Duration	25 days	Oral Virus	(-)		27,300		-
CMV	neg	Blood virus	(-)		4,400		-
5036		Day	-139		2		341
SOI	4	VCA IgM	(-)		++		
Gender	Male	VCA IgG	-		-		(++)
Duration	19 days	Oral Virus	(-)		1,000		4400
CMV	neg	Blood virus	(-)		11,400		-
5524		Day	-132		7		547
SOI	4	VCA IgM	(-)		+++		
Gender	Female	VCA IgG	-		+++		(+++)
Duration	66 days	Oral Virus	(-)		49,900		-
CMV	pos	Blood virus	(-)		60,000		-
5324		Day	-92		14		420
SOI	5	VCA IgM	(-)		+++		
Gender	Male	VCA IgG	-		-		+++
Duration	15 days	Oral Virus	(-)		2,500,000		1200
CMV	pos	Blood virus	(-)		200		-
5139		Day	-157		No sample		534
SOI	2	VCA IgM	(-)				
Gender	Male	VCA IgG	-				(+++)
Duration	15 days	Oral Virus	(-)				-
CMV	pos	Blood virus	(-)				-
5370		Day	-94		No sample		354
SOI	3	VCA IgM	(-)				
Gender	Female	VCA IgG	-				(+++)
Duration	ND	Oral Virus	(-)				700
CMV	neg	Blood virus	(-)				200
		Average	-120		0		467

** Day relative to symptom onset*
 # IgM or IgG antibodies to EBV viral capsid antigen
*** Copies/mL of viral DNA detected in oral cells or whole blood*
Values in parentheses are implied, based on values in adjacent timepoints (not shown)

Table 2-1. EBV-naïve freshman were enrolled in a prospective clinical study and followed over the course of their undergraduate education. Samples were collected at multiple intervals before, during, and after primary infection with EBV. Of 66 subjects, 10 were selected for microarray analysis. Anti-EBV antibody titers, EBV viral loads, and other patient information for the "PRE" "ACUTE" and "LATENT" timepoints are shown. SOI, Severity of illness; Duration is days of symptoms.

Primary EBV infection produces a distinct gene expression signature in peripheral blood during acute infection but not during latency

Microarray analysis was performed on peripheral blood mononuclear cells (PBMCs) from subjects described above. Each was compared with his or her own healthy pre-infection sample. Gene expression fold changes greater than 2, with a p-value of ≤ 0.05 with Bonferonni multiple tests correction were considered significant. For subjects presenting with acute IM, this analysis yielded a list of 464 genes that changed more than two fold (Figure 2-1A and Appendix Table A-1). 318 genes were upregulated and 146 genes were downregulated. Of the genes changed during acute IM, one of the largest constituent functional groups was found to be cell cycle by Ingenuity Pathway Analysis (Figure 2-1B)(Figure 1B). Likewise, pathways such as mismatch repair, DNA damage response, and ATM signaling, are associated with highly proliferative populations. Genes associated with interferon signaling were significantly enriched in the acute gene list, reflecting the host response to viral infection. Finally, pathways representing various immune functions, such as granzyme signaling, antigen presentation, NK function, and complement were enriched in the acute EBV gene list (Figure 2-1B).

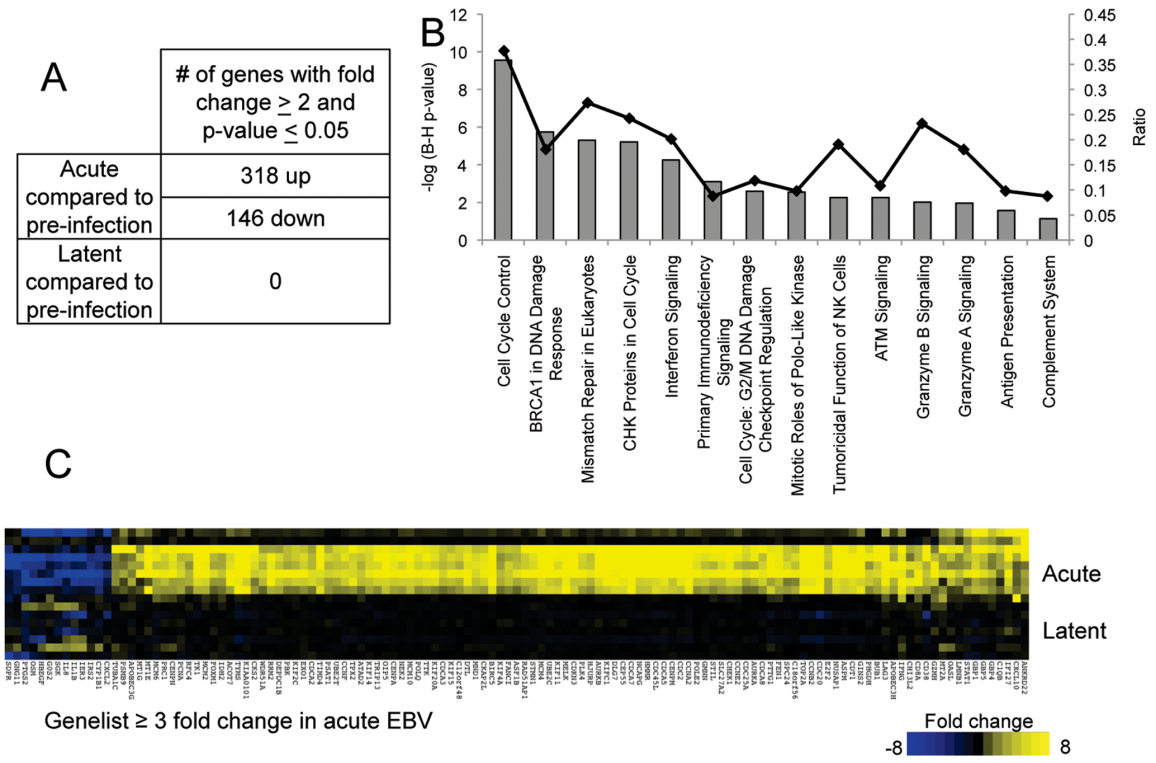


Figure 2-1. A distinct gene expression profile is apparent during acute EBV infection, but not latent infection. (A) Microarray analysis was performed on pre-infection, acute, and latent timepoints for the 10 subjects with primary EBV infection (listed in Table S1). 464 genes were shown to be significantly changed during the primary response to EBV at a fold change of ≥ 2 and a p-value ≤0.05. No genes were significantly changed during the latent phase of infection using the same criteria. (B) Ingenuity Pathway Analysis of the 464 acute genes revealed 14 pathways that were enriched amongst the genes that changed during primary EBV. These had a significant p-value (the negative log is shown) following evaluation with the Benjamini-Hochberg multiple tests correction. (C) A heatmap representation of the highest (≥ 3 fold) gene changes during the acute and latent stages of EBV infection.

Studies in mice showed that latent herpesvirus infection protected hosts from subsequent bacterial infection, suggesting that persistent infection with herpesviruses may have a beneficial role in their human hosts (127, 195). Thus, we wanted to determine if long-term gene changes were measureable in human subjects after they had acquired primary EBV. In contrast to acute infection,

subjects with latent infections yielded no significantly changed genes compared with pre-infection (Figures 2-1A/C and data not shown). This approach is highly sensitive as each subject's latent infection sample was compared with his or her own pre-infection sample. Nonetheless, there were no gene changes that met significance criteria, even *IFNG*, which was shown to be elevated in mice latently infected with a murine gammaherpesvirus. (195)

Different cell types contribute to the gene expression signature during IM

We chose to perform analysis of whole PBMCs in this study to facilitate the comparison to data published on other infections and disorders (see below). However, we still sought to understand which cellular components were representing the distinct aspects of the signature observed in acute EBV infection. Thus, we measured gene expression in sorted CD8+ T cells, natural killer (NK) cells, monocytes, and B cells (Figure 2-2A) from 4 subjects using a quantitative PCR array of 43 genes selected from the acute microarray analysis (Figure 2-3). These genes comprised the three functional groups: Type I interferon regulated genes (IRGs), type II IRGs, and cell cycle/metabolism genes.

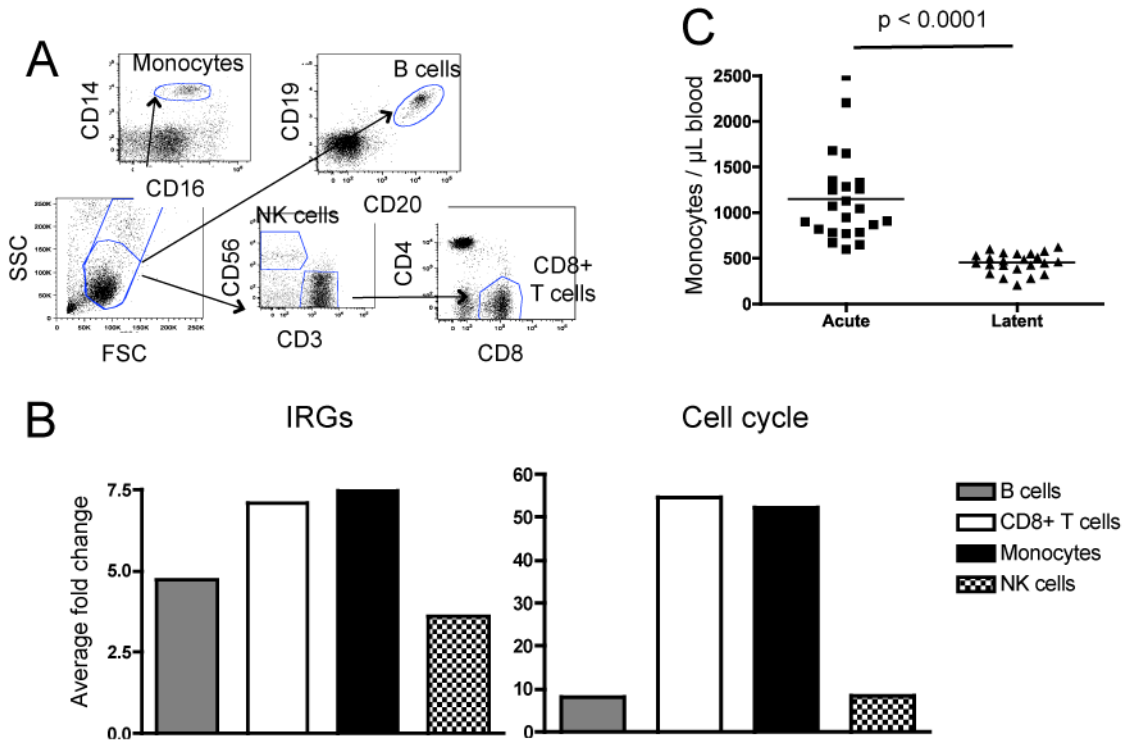


Figure 2-2. CD8+ T cells and monocytes show upregulation of key gene groups during acute infection. Quantitative PCR analysis was performed on RNA prepared from sorted cells from peripheral blood of four subjects before and during acute EBV infection. (A) This panel shows the staining and sorting strategy for separation of CD8 T cells, B cells, NK cells, and monocytes. (B) Each subject's respective cell type was compared with his or her own cell type at the preinfection time point. All acute samples were taken from subjects within a week of onset of symptoms. The average fold change of both type I and type II IRG as well as cell cycle genes for four subjects is shown. A paired t-test was used for statistical analysis. (C) Monocyte numbers were determined from complete blood counts of subjects at the acute and latent phases of EBV infection.

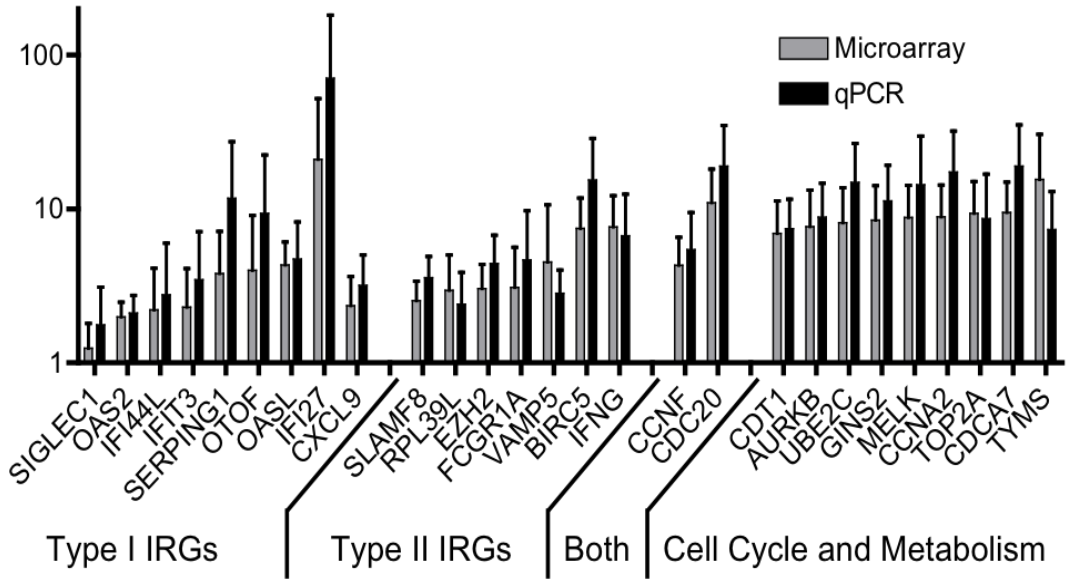


Figure 2-3. Comparison of fold changes obtained by qPCR and microarray. Average fold changes of eight patients between healthy baseline and acute sample are shown for all upregulated genes included on the qPCR array. Microarray values are shown in gray, qPCR values are shown in black. Error bars are +/- standard deviation.

All cell types displayed ≥ 3 fold upregulation of type I IRGs, suggesting that the interferon response during primary EBV infection is systemic, not limited to a particular cell type (Figure 2-2B). The cell cycle aspect of the gene expression signature, however, was highest in monocytes and CD8+ T cells. We found this result surprising given the fact that an increase in the number of monocytes had not been previously appreciated in IM literature. Complete blood counts from our subjects indicate that monocyte numbers were indeed elevated during IM (Figure 2-2C). Recent evidence has emerged showing IFNγ can induce monopoeisis, (196) thus, our data are consistent with a role for IFNγ in the proliferation of monocytes during primary EBV infection.

Human peripheral blood has been shown to have different subgroups of monocytes, which may be broadly described as inflammatory M1 monocytes or immunoregulatory M2 monocytes. (197) Given the fact that M2 monocytes are induced by IL-10 and EBV produces a viral IL-10 homolog we explored the possibility that the increase in monocytes numbers is accompanied by a shift in the M1 to M2 ratio during IM. However, no major change in expression of CD163 or HLA-DR (commonly used to distinguish M1 and M2) was found on monocytes during acute infection (data not shown). Thus, although monocytes are increased during acute infection, they may represent a heterogeneous mix of cells that does not fit the established M1/M2 paradigm.

Comparison of EBV to other viral infections and SLE

We compared our data on EBV with published data sets from five other acute viral infections including influenza A virus (Flu), human rhinovirus (HRV), respiratory syncitial virus (RSV), (198) dengue fever virus (DENV), (199) and yellow fever virus vaccine strain (YFV). (200) Additional comparison was performed against samples from patients with the autoimmune disease SLE, because EBV had been suggested to be an etiologic agent of SLE(201) and SLE patients have been found to express IRGs. (202) Data were also compared with subjects who were administered polyinosinic:polycytidylic acid (Poly IC), as an inducer of a classic type I interferon response. (203) A heatmap of the EBV gene set with a fold change (FC) ≥ 3 provided a good visual representation of this comparison (Figure 2-4), although a more complete gene list (FC ≥ 2) can be

found in Appendix Table A-1. Young adults presenting with acute EBV infection have a distinct gene expression pattern from those with other acute viral infections, SLE, or subjects administered Poly IC (Figure 2-4). Four main points are apparent from the heatmap:

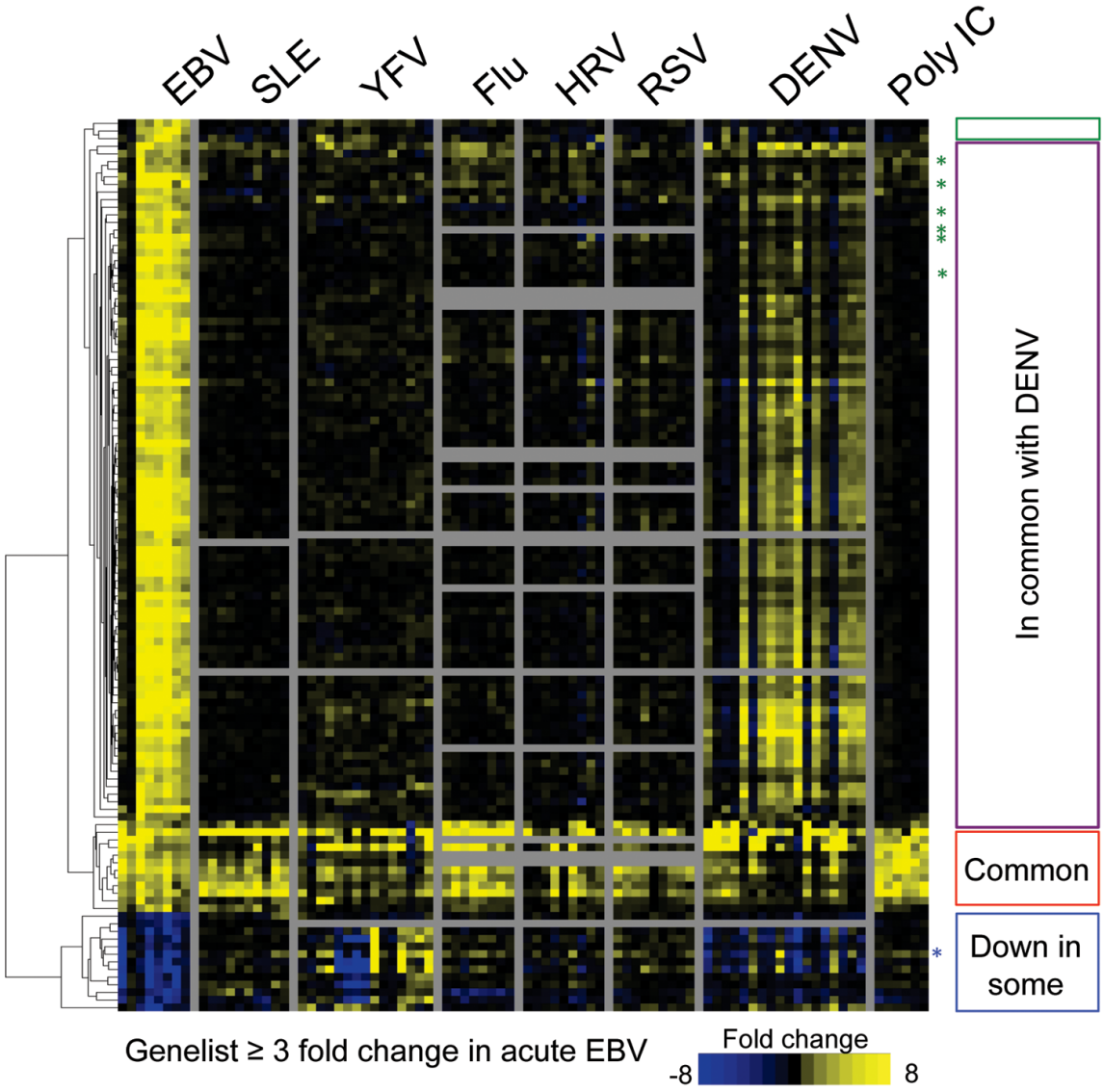


Figure 2-4. Primary Epstein-Barr virus infection causes distinct expression patterns in comparison to other interferon driven responses. Heatmap shows a list of the genes up/down regulated 3-fold during acute infection with EBV in comparison to subjects with other viral infections (YFV, Flu, HRV, RSV, and DENV), subjects injected with Poly IC, or patients with SLE. The color intensity represents fold changes in gene expression in comparison to either

each subjects' own healthy baseline (EBV, Flu, HRV, RSV, and Poly IC) or in comparison to healthy controls (SLE and DENV). Data is arranged as a hierarchical clustering of genes. The genes shown were derived from analysis of EBV subjects (fold change ≥ 3 and met a statistical significance cutoff of p-value of ≤0.05 with Bonferonni multiple-tests correction, please see Table S2 for fold change ≥ 2). Each column represents a single subject. Horizontal gray bars indicate that the gene of interest was not present in the analyzed dataset used for comparison. Vertical gray bars separate disease groupings. Colored boxes on the right represent specific clusters of genes discussed in the text: green box and green asterisks--EBV unique, purple box--genes in common between EBV and DENV, red--genes upregulated in all situations, blue--genes down-regulated in EBV and some other situations.

IRGs are induced in all viral infections. The greatest similarity between EBV and other acute viral illnesses was found amongst the genes that are outlined in the red box in Figure 2-4 Figure 3 (also listed in Appendix Table A-1). These genes are composed almost exclusively of IRGs. IRGs were defined using the online database Interferome and by comparison to PBMCs stimulated by IFN *in vitro* (data not shown). Not surprisingly, such genes were also upregulated by Poly IC, and were a dominant element of the SLE signature (Figure 2-4). (202) Interestingly, only a minority, 21% of the EBV gene signature was comprised of IRGs. This is markedly lower than Flu and YFV, where 48% and 65%, respectively, of the gene changes were IRGs (Figure 2-5). Further analysis via classification of type I IRGs and type II IRGs, showed there was fairly equal representation between the two IFN types for EBV. Flu and YFV, however, were dominated by type I IRGs, with more than 80% being regulated by type I IFN (Figure 2-5). Thus, while primary EBV exhibited marked upregulation of IRGs, they did not represent a dominant aspect of the signature, and reflected an

enrichment of a type II IFN response in addition to the more typical type I IFN response.

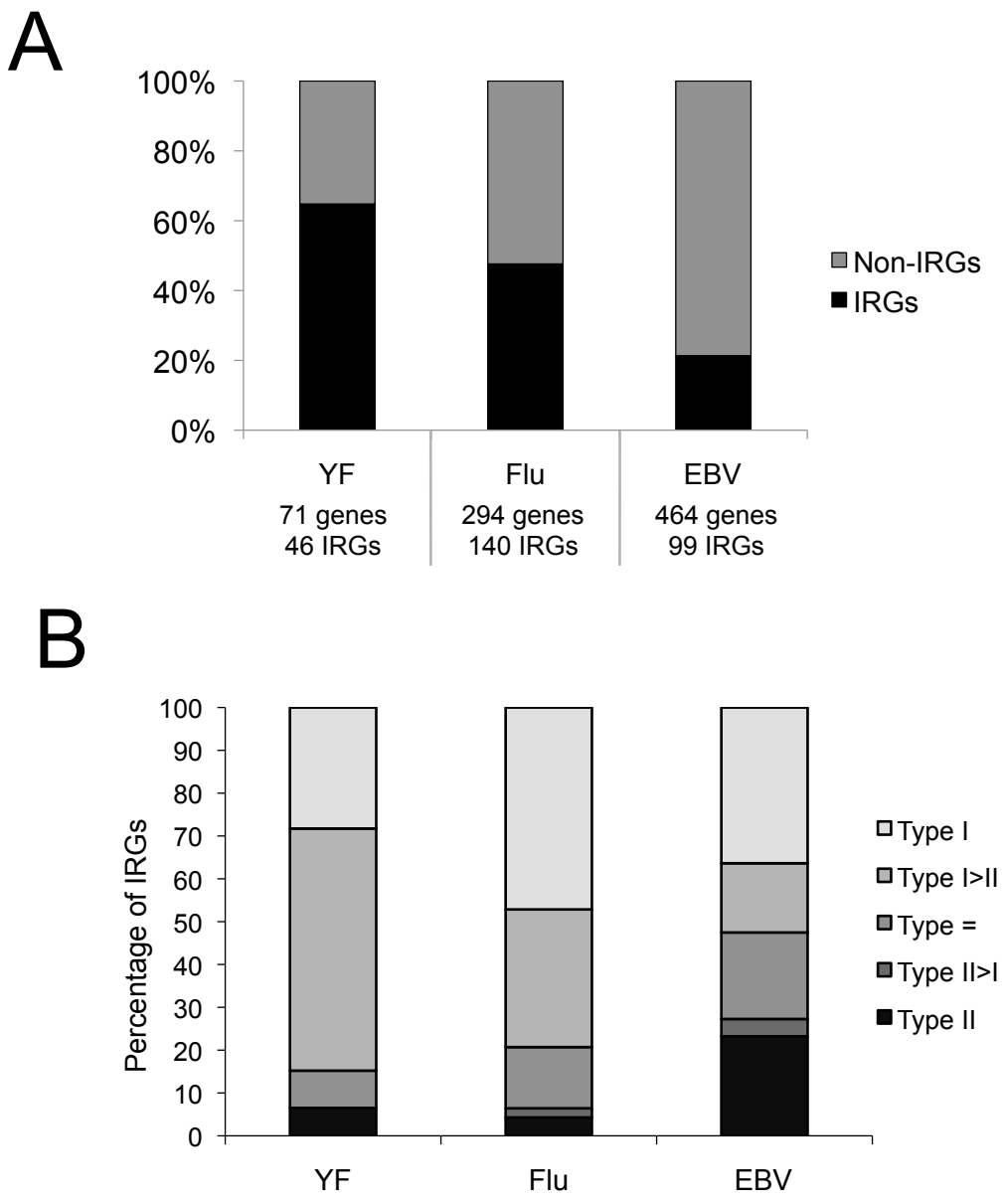


Figure 2-5. EBV demonstrates a bias toward a type II IRG expression pattern. (A) Genes upregulated during acute viral infection were divided into IRG and non-IRG subsets. The number of genes that changed significantly are listed for each infection. The proportion of IRG and non-IRG are shown in the bar graph. (B) Only genes classified as IRG were used for this analysis. IRGs were separated into groupings based on relative upregulation by type I and type II IFN in PBMC stimulated with IFN in vitro.

EBV unique genes. The heatmap in Figure 2-4 also shows a set of genes increased in primary EBV infection but not other infections (Figure 2-4), green box and green asterisks). We defined a total of 59 genes that were “EBV unique” in comparison to all other acute viral infections examined using a 2-fold increase cutoff for EBV (Appendix Table A-2). Given this finding, we wondered if primary EBV infection could be distinguished from other acute common viral illnesses. We conducted a small, blinded analysis of clinical samples. Analysis of 43 genes correctly distinguished acute EBV illness from non-EBV viral illnesses (Figure 2-6).

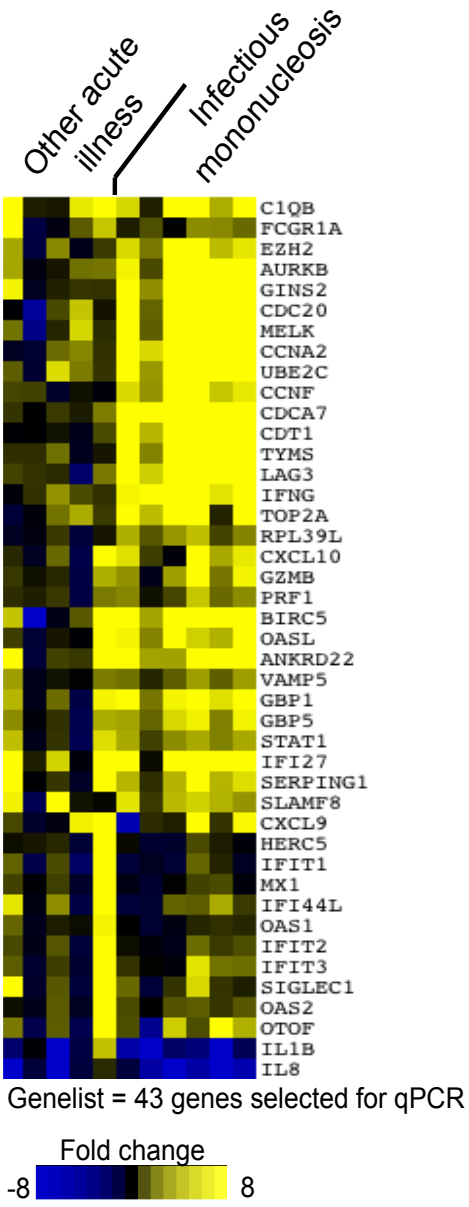


Figure 2-6. Gene set analysis can successfully segregate the EBV signature from other acute illnesses. Gene expression of the indicated genes was determined by qPCR on 11 symptomatic subjects. Heat map indicated fold change from each subject's own baseline. K-means clustering was performed on 8 blinded samples and three known EBV samples. Analysis was performed with three clusters and 10,000 runs. Three samples were obtained from subjects presenting with acute primary EBV infection. Five additional samples were collected from subjects presenting with other undefined acute illnesses. The symptomatic primary EBV infection signature is characterized by high cell cycle gene expression, low type I IRG expression, and high IFNG expression.

Response to EBV shares similarity to Dengue. From this comparison, we also observed that EBV shares little overall similarity to other acute infections with the exception of DENV. Almost 60% of the genes upregulated ≥ 3 by EBV, and nearly all of the downregulated genes were also similarly up/down regulated in a majority of DENV patients (Figure 2-4, purple and blue boxes). A possible pitfall to comparing disparate human infections and conditions is that sample timing may be different in infections with different incubation times. Nonetheless, the large group of “in common with DENV” genes was not upregulated at any time point even up to 28 days following YFV or Poly IC (Figure 2-7). Thus, upregulation of these genes seems peculiar to EBV and DENV.

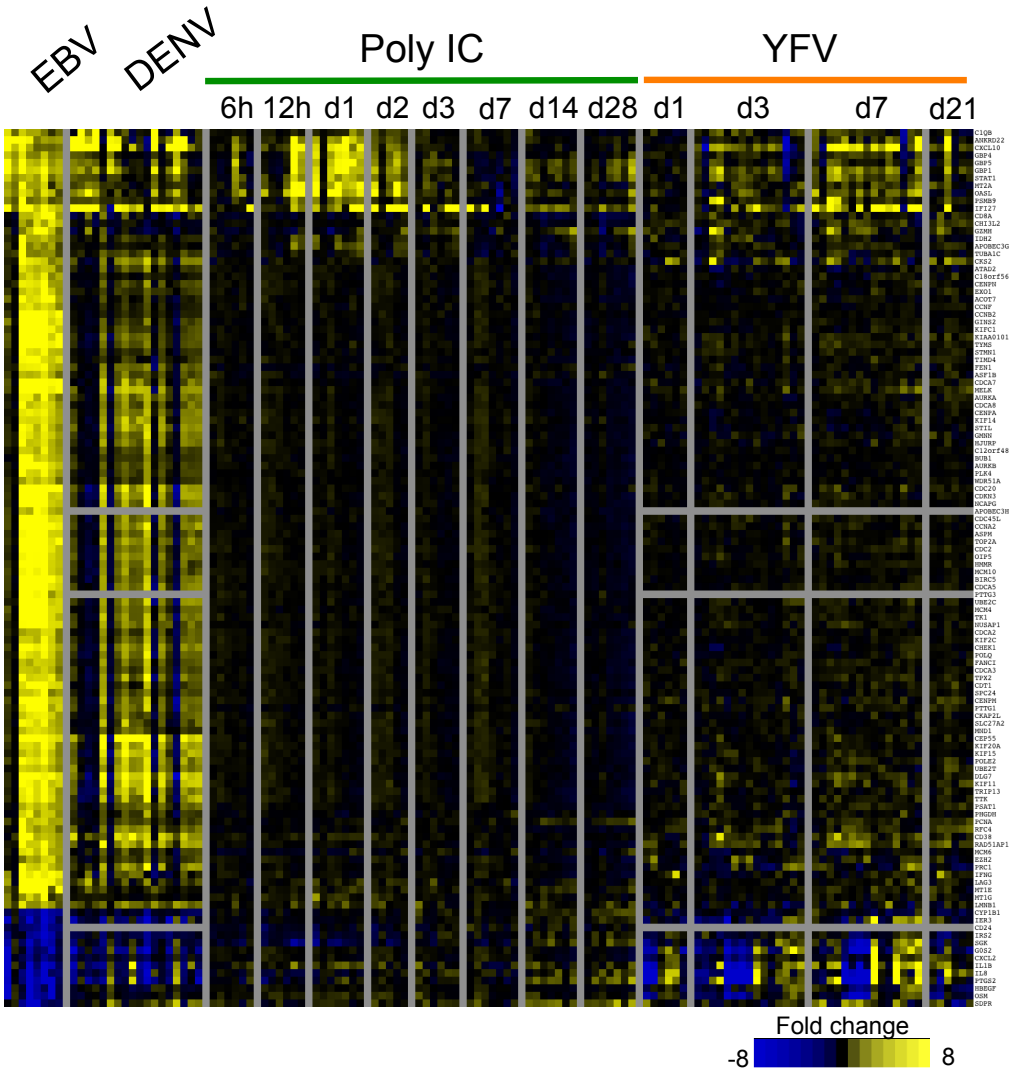


Figure 2-7. Most EBV/DENV induced genes were not increased at any timepoint following YFV vaccination or Poly IC treatment. The genes shown were derived from analysis of EBV subjects (fold change ! 3 and met a statistical significance cutoff of p-value of " 0.05 with Bonferonni multiple-tests correction). Gene expression is shown in comparison to subjects with other viral infections (YFV and DENV) or subjects injected with Poly IC. Data is arranged as a hierarchical clustering of genes. Each column represents a single subject. Horizontal gray bars indicate that the gene of interest was not present in the analyzed dataset used for comparison. Vertical gray bars separate disease or time groupings. The color intensity represents fold changes in gene expression in comparison to either each subjects' own healthy baseline (EBV, YFV and Poly IC) or in comparison to healthy controls (DENV).

Genes down-regulated after EBV. The heatmap in Figure 3 also identifies a discrete set of genes downregulated in acute EBV infection (Figure 2-4, blue box). Many of these genes are inflammatory or stress response genes expressed by monocytes, including *IL1B, IL8, MIP2A*, and *OSM* (Appendix Table A-3). The down-regulation of *IL1B* and *IL8* was confirmed by real-time PCR in 70 timepoints from 52 subjects (data not shown). This downregulation is particularly surprising for *IL1B*, which is considered a type I IRG and therefore one might expect it would be upregulated during viral infection. Indeed, *IL1B* was generally upregulated during antiviral responses, including subjects with Flu, HRV, and RSV, and subjects treated with Poly IC or SLE patients (blue asterisk in Figure 2-4).

EBV and DENV resemble inflammatory syndromes

Due to the surprising difference between the EBV/DENV signature and that observed in other acute viral infections, we theorized that perhaps the signature would more closely resemble that observed during inflammatory syndromes. To this end we examined microarray data obtained from subjects with familial hemophagocytic lymphohistiocytosis (FLH) (204) or subjects presenting with systemic onset juvenile arthritis (sJIA) who were later confirmed to have subclinical macrophage activation syndrome (MAS). (205) Both of these syndromes may be classified under the larger umbrella of hemophagocytic diseases, but are distinct in their origins. FLH is most commonly caused by mutations associated with degranulation genes, especially perforin (*PRF1*) and

the syntaxins. The genetic basis of MAS is less well understood but the vast majority of patients first present with sJIA. (206) Indeed, many of the gene changes shared between EBV and DENV were also found in patients with these inflammatory disorders (Figure 2-8). This suggests that the host defense to EBV and DENV more closely resembles uncontrolled inflammation that it does during other antiviral response. This would help explain the lack of similarity between the EBV response and what is observed in other acute viral infections or an IFN associate autoimmune disease.

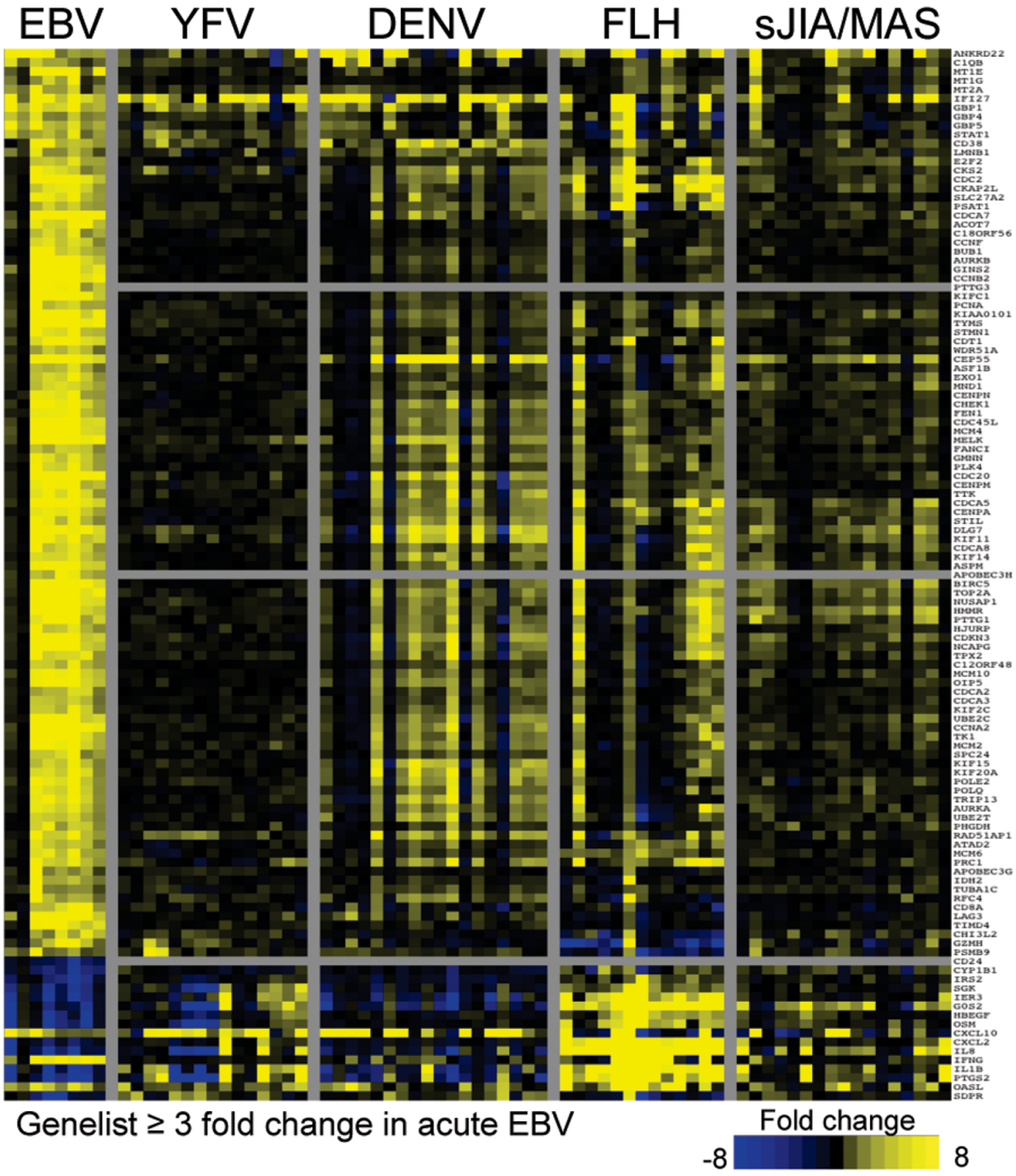
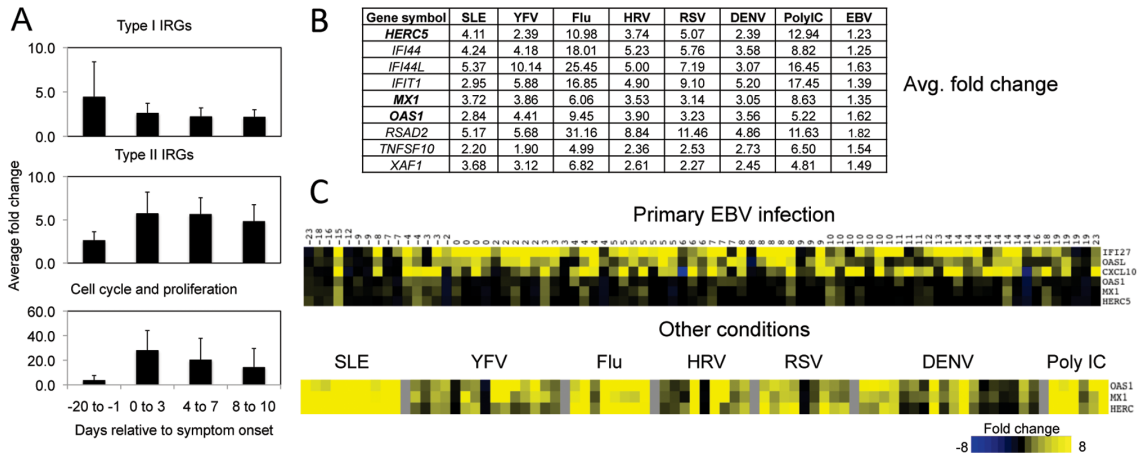


Figure 2-8. EBV has high similarity to DENV and inflammatory syndromes. This heatmap compares the genes that change during primary EBV with DENV, and two syndromes: juvenile idiopathic arthritis with macrophage inflammatory syndrome (sJIA/MAS) and familial lymphocytic histiocytosis (FLH). The fold changes in gene expression are in comparison to either each subjects' own healthy baseline (EBV) or healthy controls (DENV, sJIA/MAS and FLH). Genes shown had a fold change ≥ 3 in acute EBV and met a statistical significance cutoff of p-value of ≤0.05 with Bonferonni multiple-tests correction.

Type I and type II IFN response kinetics differ

Although we cannot know precisely when subjects were infected with EBV, we were able to "stage" the subjects relative to the date of symptom onset. In the heatmaps shown, the subjects are ordered according to their symptom onset date. It is apparent that set B genes (IRGs) were upregulated in two of the pre-symptomatic subjects, but the other major aspect of the signature was absent (Figure 2-7). To determine if this reflected temporally distinct aspects of the signature, we extended our analysis to multiple timepoints from a larger number of subjects. We employed the PCR array of 43 genes described above, representing genes in three main functional groups: Type I IRGs, type II IRGs, and cell cycle/metabolism. With this assay, we extended our analysis to include data from 70 different timepoints between -23 days and 20 days relative to symptom onset, comprising samples from 50 subjects. The trend from these analyses suggests that on average, type I IRG changes occur earlier during infection (including during the incubation period) than either type II IRG or cell cycle genes (Figure 2-9A).



Gene symbol	SLE	YFV	Flu	HRV	RSV	DENV	PolyIC	EBV
HERC5	4.11	2.39	10.98	3.74	5.07	2.39	12.94	1.23
IFI44	4.24	4.18	18.01	5.23	5.76	3.58	8.82	1.25
IFI44L	5.37	10.14	25.45	5.00	7.19	3.07	16.45	1.63
IFIT1	2.95	5.88	16.85	4.90	9.10	5.20	17.45	1.39
MX1	3.72	3.86	6.06	3.53	3.14	3.05	8.63	1.35
OAS1	2.84	4.41	9.45	3.90	3.23	3.56	5.22	1.62
RSAD2	5.17	5.68	31.16	8.84	11.46	4.86	11.63	1.82
TNFSF10	2.20	1.90	4.99	2.36	2.53	2.73	6.50	1.54
XAF1	3.68	3.12	6.82	2.61	2.27	2.45	4.81	1.49

Figure 2-9. Type I IRGs are slightly enriched before onset of symptoms, but some IRGs show no change. (A) Superarray analysis by qPCR was performed on 70 timepoints from 52 subjects to assess the time course of gene expression. Average fold changes for three groups of genes (Type I IRG, Type II IRG, and Cell Cycle as defined in Figure S1) were determined for samples collected within the indicated time blocks. Fold changes are compared with healthy baselines. Error bars show the standard deviation. All three gene groupings had a p-value <0.05 when evaluated by ANOVA. (-20 days to -1 days N = 11; 0 days to 3 days N = 12; 4 days to 7 days N = 17; 8 days to 10 days N = 16). (B) The table shows the average fold change for nine select type I IRGs derived from microarray analysis for SLE, YFV, Flu, HRV, RSV, DENV, Poly IC, and EBV. (C) Heatmaps showing fold change as determined by qPCR for *OAS1*, *MX1*, and *HERC5* at multiple timepoints in EBV infection (top panel) or in other situations (bottom panel).

EBV fails to induce key interferon response genes

While a type I interferon response clearly occurs during EBV infection, we were surprised to observe that *MX1*, which is a classic IFN induced GTP-binding protein, (207) was not upregulated in any of the primary EBV subjects. Upon further examination, we defined nine IRGs that were upregulated in all other viral infections, with Poly IC, and in SLE patients; but not in the EBV subjects (Figure 2-9B). To determine if we may have missed an early spike in gene expression as suggested from the analysis above, we selected three of these *MX1*, *OAS1*, and

HERC5 and performed real-time PCR on 70 timepoints from 50 subjects. As expected, these genes were upregulated in every other acute infection data set examined and in SLE (Figure 2-9C bottom rows). However, none were upregulated more than two fold in any of the pre-symptom or post-symptom timepoints we examined (Figure 2-9C). Selective antagonism of specific IRGs or interferon signaling is common in viral infections. (208, 209) However, one would typically expect this to only be observed in virally infected cells (in our case, relatively rare circulating B cells). Instead the selective antagonism of IRGs we observed is occurring in the context of a systemic interferon response. Thus, we favor the hypothesis that EBV produces a soluble molecule that selectively antagonizes IRG gene expression in uninfected cells.

The IRG signature of SLE is not similar to EBV

EBV has been suggested to be involved in the pathogenesis of SLE. (210) A significantly higher proportion of SLE patients are infected with EBV than the general population (211, 212) and they have higher viral loads and antibody titers. (213, 214) Some SLE patients also have high IFN signatures. (202) Thus, it was hypothesized that the interferon response may be driven by EBV (210) and EBV derived nucleic acids could fuel type I IFN production via plasmacytoid dendritic cells. (215) We sought to investigate this link at the gene expression level. Patients with high IFN signatures (202) were compared with healthy controls using the same statistical criteria described for EBV above. Interestingly, we found that SLE had the least similarity to EBV as demonstrated by the low

Pearson r-value (Figure 2-10). Conversely, both Flu and Poly IC had high levels of correlation. In particular the nine IRGs that are selectively antagonized during EBV infection are strongly upregulated in SLE patients (Figure 2-9B). Thus, our data do not support the notion that EBV reactivation contributes to the interferon gene signature in SLE.

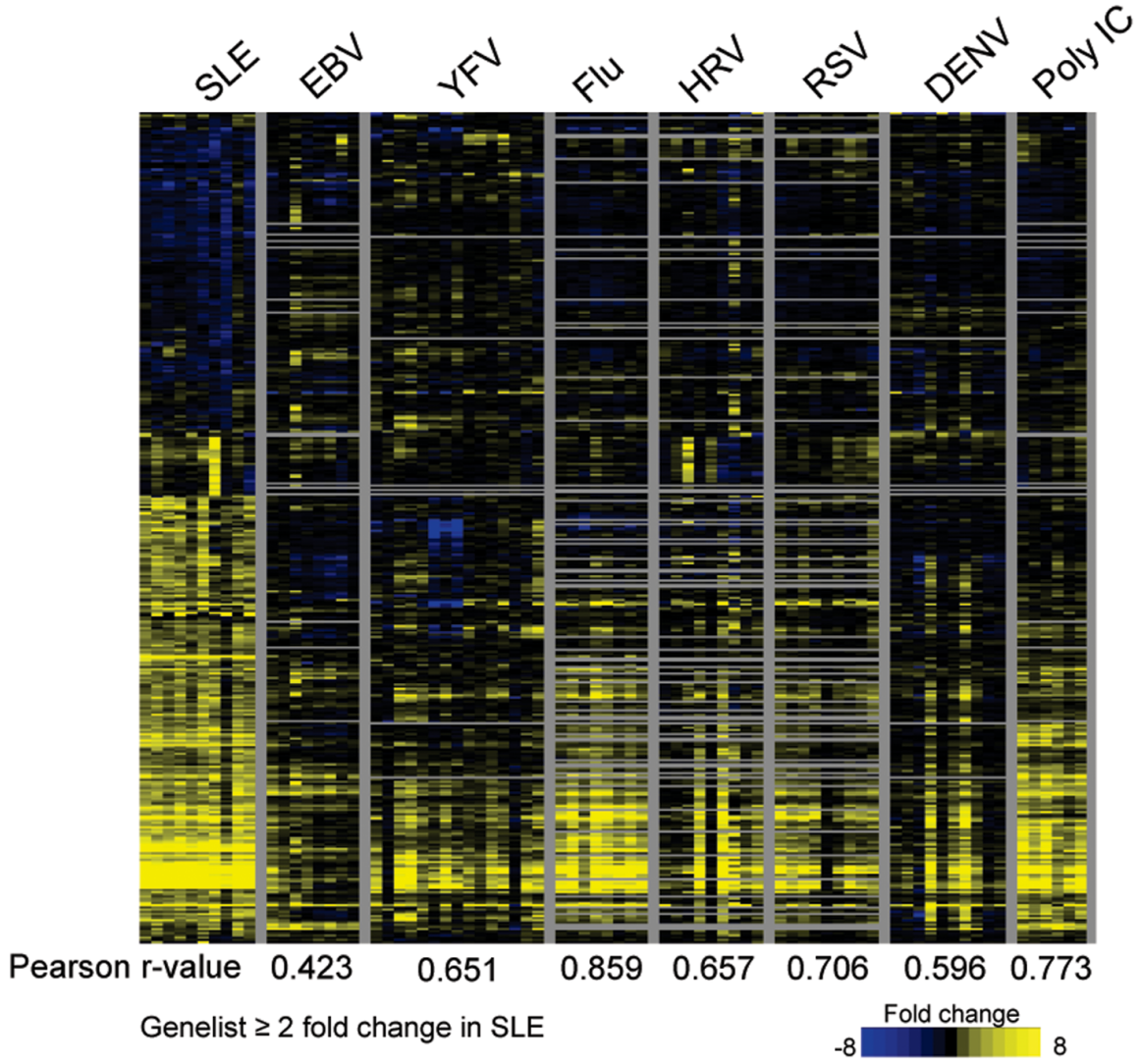


Figure 2-10. SLE has high similarity to influenza and Poly IC but not EBV or DENV. The heatmap lists the genes that are up/down regulated ≥ 2 fold in SLE patients compared with subjects with viral infections or who were injected with Poly IC. The fold changes in gene expression are in comparison to healthy controls (SLE and DENV) or to each subjects' own healthy baseline (EBV, YFV, Flu, HRV, and RSV). Pearson r-values indicating degree of similarity between SLE and each of the other conditons are shown at the bottom of the heatmap.

Type II IRG expression correlates with CD8 T cell expansion in primary EBV expression

Finally, we investigated whether or not there was a correlation between gene expression and severity of illness during acute infection. A hallmark of

infectious mononucleosis is the dramatic expansion of CD8 T cells. Indeed, both the total number of CD8 T cells/ml blood or the ratio of CD8 to CD4 T cells in the blood provided the best predictor of disease severity in a large prospective study. (14) Thus, using data from the qPCR superarray approach described above, we examined the correlation between gene expression and CD8 lymphocytosis. We found that expression of type I IRGs individually, or as a group, did not correlate strongly (Figure 2-11). This was true even when we limited the analysis to specific time windows where type I IRG gene expression was highest (data not shown). On the other hand, upregulation of type II IRG as a group did correlate positively (Figure 2-11). The upregulation of three individual genes correlated very significantly with CD8 lymphocytosis. These three: *OASL*, (Pearson $r = 0.6059$, $p < 0.0001$) *TYMS* ($r = 0.5019$, $p = 0.0006$), and *SLAMF8* ($r = 0.6028$, $p < 0.0001$), are all upregulated by IFNγ consistent with the known effects of IFNγ when given as therapy (216, 217). Interestingly, *OASL* in particular has been hypothesized to cause fatigue through antagonism of the thyroid receptor (218). Altogether these results reinforce the concept that many IM symptoms are immunopathologic, resulting from high levels of IFNγ produced by either innate or adaptive immune cells.

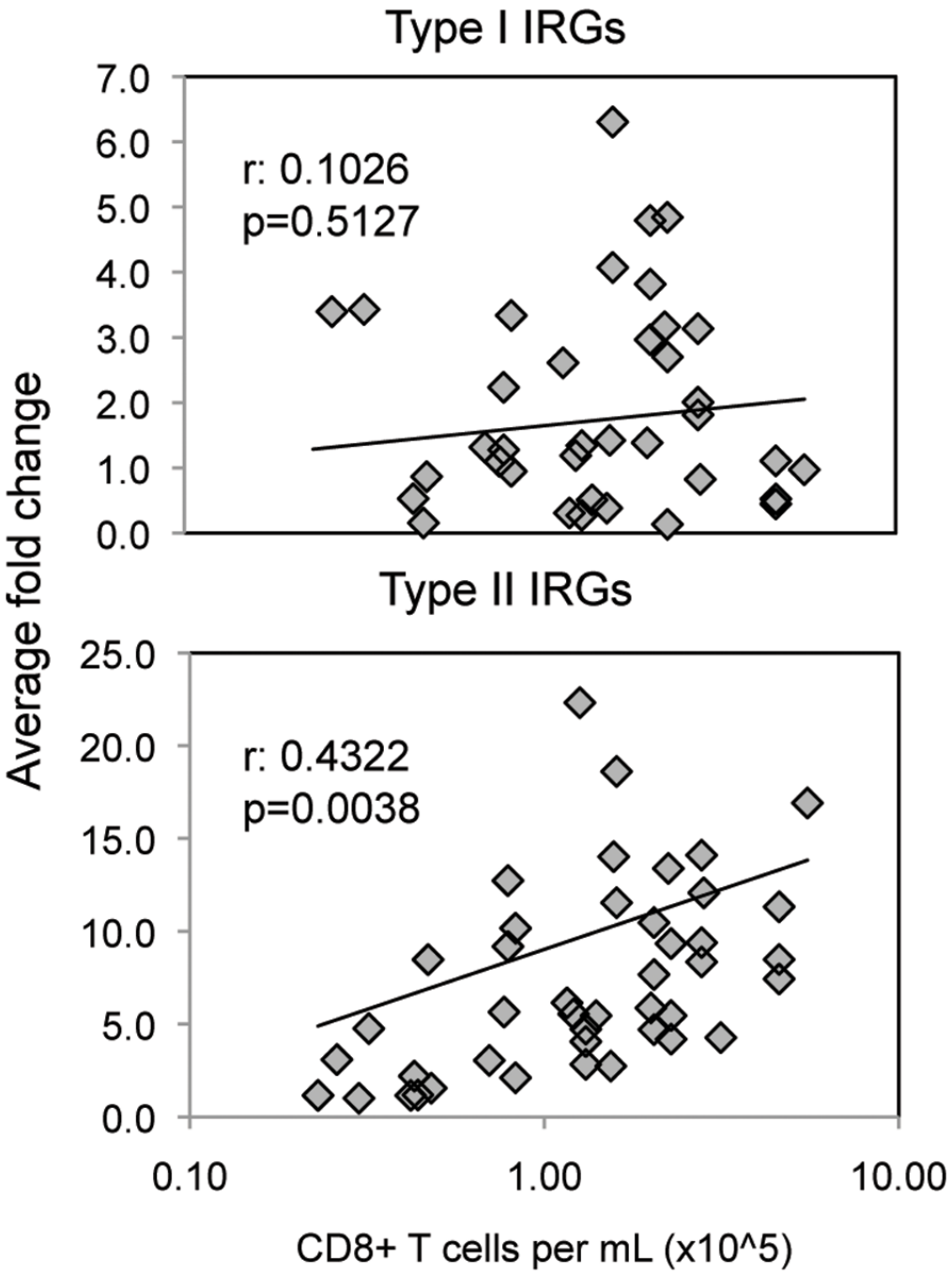


Figure 2-9. SLE has high similarity to influenza and Poly IC but not EBV or DENV. The heatmap lists the genes that are up/down regulated ≥ 2 fold in SLE patients compared with subjects with viral infections or who were injected with Poly IC. The fold changes in gene expression are in comparison to healthy controls (SLE and DENV) or to each subjects' own healthy baseline (EBV, YFV, Flu, HRV, and RSV). Pearson r-values indicating degree of similarity between SLE and each of the other conditons are shown at the bottom of the heatmap.

Discussion

This study provides the first gene expression profiling of a natural herpes virus infection in healthy human subjects. One notable aspect of the EBV gene expression profile was the striking upregulation of genes related to cell cycle and proliferation. While proliferation of cells is likely to be part of the immune response to any pathogenic virus, what is striking is the magnitude and duration of these gene expression changes, which allow them to be observed at the level of whole blood gene expression profiling. Most other viral infections did not display significant upregulation of these genes at any time point. The exception was DENV. Indeed the shared profile of EBV and DENV was also observed in hemophagocytic syndromes, emphasizing the exaggerated inflammatory response that ensues in these viral infections. Although other potentially interesting comparisons would be to DNA viruses, particularly CMV, which can cause IM, there are currently no data available on gene expression during primary infection in humans.

In contrast to the readily apparent gene signature in acute infection, we were surprised to discover that there were no consistently detectable gene expression changes in human blood during latent infection, despite the sensitivity of our approach. EBV is known to persist in the B cell compartment, albeit at quite low levels (1-50 copies per million B cells). (219) The virus persists in a very “quiet” state, likely expressing viral RNAs (EBERs) but little or no viral protein products. (220) Nonetheless, immunosuppression can result in viral recrudescence with sometimes fatal consequences, (221, 222) thus EBV is

presumed to be under constant active immune surveillance. However, EBV is unlikely to be the only chronic viral infection present in our subjects. Up to a dozen chronic viral infections can be common in humans, including the related herpesvirus CMV (5) although it should be noted that the majority of our subjects (88%) were CMV negative and remained so throughout the study.

Through comparison of the EBV signature to gene expression profiles of other acute viral infections in healthy individuals, we were able to identify the common and unique aspects of the response to EBV. While the response to all infections involved upregulation of IRGs, the response to EBV was notably lacking the upregulation of a subset of nine key IRGs. These particular genes are of interest as potential targets of viral antagonism. In addition we observed a striking downregulation of *IL1B*, which was surprising given that monocytes are activated and proliferating during acute EBV infection. Recent data suggested IL-1β may be downregulated by type I IFN in human monocytic cells *in vitro*. (223) However, it is not clear why *IL1B* would be downregulated by IFN in EBV but upregulated by IFN in most other viral infections. Another possibility was suggested by a recent report, which showed that EBV encodes a microRNA (BART15) that targets the NLRP3 inflammasome, and exosome mediated secretion of this miRNA resulted in downregulation of IL-1β in bystander (uninfected) monocytes *in vitro*. (224) Intriguingly, *IL1B* was also down regulated in most DENV patients and in about half of subjects given the YFV vaccine (Figure 2-7). This disparate regulation may be interesting in the context of monopoeisis and an environment of high IFNγ production. In patients

administered G-CSF, immature monocytes entering the blood stream from the bone marrow bear surface bound IL-10. (225) In the presence of IL-10, monocytes that have become activated by IFNγ produce much less *IL1B*. (226) Furthermore, EBV encodes a viral IL-10 homologue (vIL-10) (227), suggesting another possible explanation for our observation. Further study will be required to determine the mechanism and significance of the profound *IL1B* downregulation. We hypothesize that the lack of upregulation of *IL1B* and key IRGs may allow this particular herpesvirus to avoid elicitation of adaptive immunity for many weeks, which may aid in establishing latency and provide an explanation for the long incubation period prior to symptoms.

Materials and Methods

Ethics statement

All participants gave written informed consent and the University of Minnesota Institutional Review Board approved all protocols used.

Design of prospective study

We recruited 546 healthy undergraduate volunteers from the University of Minnesota residence halls in 2006 and 2007 as recently described. (194) EBV naïve subjects were identified as those lacking IgG antibodies against EBV viral capsid antigen (EBV VCA IgG) and EBV nuclear antigen 1 (EBNA1). Of the 202 eligible EBV-naïve subjects, 143 (71%) were enrolled in the prospective study. For enrolled participants, blood and oral washings were 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 asked to come to the clinical virology research clinic for both a physical exam and laboratory-confirmation of primary EBV infection via Monospot, EBV serology, and viral titer in the oral cavity or blood. 66 subjects experienced primary infection (46%), of which only 6 were asymptomatic. (194) Primary EBV infection was defined as a positive EBV antibody test and the presence of EBV DNA in the blood and/or oral compartment of a subject who was previously negative for both EBV antibodies and EBV DNA. All participants continued pre-scheduled follow-up visits after seroconversion.

Samples 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. Blood 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.

RNA extraction

For each sample, 1-2 x 10^6 PMBCs were used for each RNA extraction. Cells were first homogenized using QIAshredder columns (Qiagen) per the manufacturer's instructions. RNA was then extracted using RNeasy kit (Qiagen) with on column DNase step (Qiagen) per the manufacturer's instructions. RNA was then quantified using a Nanodrop 2000/2000c spectrophotometer (Thermo Scientific) and kept frozen at -80°C.

Microarray

200ng of RNA per sample was amplified for microarray analysis using the Illumina TotalPrep RNA Amplification Kit (Ambion) per the manufacturer's instructions. Tagged transcripts were then sent to the microarray facility of the BioMedical Genomics Center at the University of Minnesota for hybridization to an Illumina Sentrix BeadChip HumanRef-8 v3. Preinfection and latent samples were hybridized to Illumina HumanHT-12 v4 Expression Beadchips.

Microarray analysis and heatmap generation

Raw data from (198-200, 202-205) were obtained from the GEO database and analyzed independently as described. Signal values from all microarrays were imported into the microarray analysis program GeneSpring (Agilent). Signal values were normalized by percentile and filtered to eliminate noise from the data. Fold changes were calculated as the difference between acute infection samples and baseline (in the event that the subjects had baselines) or healthy controls (for clinical studies with no available baselines). Studies with before and after samples were subjected to a paired *t*-test with Bonferonni multiple tests correction. Studies which used healthy controls were subjected to an unpaired unequal variance *t*-test (Welch) with Bonferonni multiple tests correction. Log_2 transformed fold changes were exported from GeneSpring into a *.txt file. These values were imported into the open source program Cluster 3.0, (228) which clustered the genes hierarchically using a Pearson non-averaged correlation and average linkage. Heatmaps were then visualized using the program Java Treeview. (229)

Ingenuity Pathway Analysis

Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software (Ingenuity System). Gene sets were screened to limit the number of genes utilized for analysis. For the acute gene set, the 464 significantly changed genes previously described were used. 53 genes with potential long-term

changes were used from the latent analysis. Log_2 transformed fold changes for all relevant genes were imported into the software using their Illumina probe identifiers and were then associated with their appropriate gene symbol by IPA. The software then used these genes and their fold changes to determine which gene sets had statistically significant importance in each of the cases. Canonical pathways were assigned based on the number of genes present in our geneset for each pathway, the level at which these genes were up or down regulated, and the likelihood these trends would occur by chance by using the multiple test correction Fisher's exact test. Results are reported in this work as the Benjamini-Hochberg p-value or ratio.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed on whole chip acute and latent infection microarray data using GSEA software (Broad Institute). Analysis was performed per Broad Institute instructions.

Fluorescence activated cell sorting

Frozen PBMCs from baseline and acute IM timepoints from 4 subjects were thawed and stained to be sorted into four different subsets: CD8 T cells (CD3+, CD56-, CD8+), NK cells (CD56+, CD3-), B cells (CD19+, CD20+), and monocytes (CD14/CD16). Subsets were sorted into 1.5 ml tubes pre-coated with media using a FACS Aria under BSL-2 sorting conditions. Cells were washed with PBS and RNA was extracted as described above.

cDNA synthesis and PCR analysis using SuperArray

100ng of RNA was used with the SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen) to generate cDNA. Samples were then stored at -20°C. 43 genes were selected for analysis by PCR from a larger list of changed genes in IM subjects and other acute viral infections that comprised relevant functional groupings as assessed by IPA. 384-well SuperArray plates pre-coated with primers for the desired 43 genes plus 5 controls were obtained as a custom order from SABiosciences. 10 µL of cDNA per subject was used with RT2 Real-time SYBR green/Rox PCR master mix (SABiosciences) for qRT-PCR analysis. Products were detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The genes ACTB, B2M, and RPL13A were used as housekeeping genes during the calculation of fold changes as they had previously been shown not to change significantly during acute EBV infection (data not shown). Fold changes were calculated as: 2^(Δ Acute Housekeeping Control – Baseline Housekeeping Control)/2^(Δ Acute Gene of Interest – Baseline Gene of Interest).

Blinded study and K-means clustering

Subject samples for the blinded gene signature study were chosen by H.H.B Jr and RNA extraction was performed by O.A.O to ensure S.K.D was blinded to sample identity. cDNA synthesis, qRT-PCR and analysis were performed by S.K.D. Sample cycle thresholds were imported into Cluster 3.0 as a text file and

a K-means clustering was performed on the samples with 3 groupings and 10,000 iterations to provide a sufficient number of successful solutions from the algorithm.

Statistical analysis

Except for where Genespring (Agilent) or IPA (Ingenuity Systems) was used, most other statistical analysis was performed using Prism software (Graphpad). MedCalc (MedCalc Software) was used for comparison of Pearson correlation coefficients. Comparisons between groups were performed with either an unpaired two-tailed *t*-test or a one-way ANOVA with a p-value less that 0.05 as the cutoff for statistical significance. Pearson correlation coefficients were generated with Prism by comparing pairs of relevant groupings.

Accession numbers

Gene expression data is available from the Gene Expression Omnibus. The data are part of superseries GSE45924 composed of two subseries, GSE45918 and GSE45919.

Chapter 3

The incubation period of primary Epstein-Barr virus infection: viral dynamics and immunologic events

Abstract

Epstein-Barr virus (EBV) is a human herpesvirus that causes acute infectious mononucleosis and is associated with cancer and autoimmune disease. While many studies have been performed examining acute disease in adults following primary infection, little is known about the virological and immunological events during EBV's lengthy 6 week incubation period owing to the challenge of collecting samples from this stage of infection. We conducted a prospective study in college students with special emphasis on frequent screening to capture blood and saliva samples during the incubation period. Here we describe the viral dissemination and immune response in the 6 weeks prior to onset of acute infectious mononucleosis symptoms. While virus is presumed to be present in the oral cavity from time of transmission, we did not detect viral genomes in the oral wash until one week before symptom onset, at which time virus was present in high copy numbers, suggesting loss of initial viral replication control. In contrast, using a sensitive nested PCR method, we detected virus at low levels in blood about 3 weeks before symptoms. However, high levels of EBV in the blood were only observed close to symptom onset –coincident with or just after increased virus in the oral cavity. The early presence of virus in the blood, even at low levels, correlated with a striking decrease in the number of circulating plasmacytoid dendritic cells well before symptom onset, which remained depressed throughout convalescence. On the other hand, natural killer cells expanded only after symptom onset. Likewise, CD4+ Foxp3+ regulatory T cells decreased two fold, but only after symptom onset. We observed no substantial

virus specific CD8 T cell expansion during the incubation period, although polyclonal CD8 activation was detected in concert with virus increasing in the blood and oral cavity, possibly due to a systemic type I interferon response. This study provides the first description of events occurring in vivo during the incubation period of natural EBV infection in humans, and definitive data upon which to formulate theories of viral control and disease pathogenesis.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus. As with all herpesviruses, EBV causes lifelong infection in its host. Infection is associated with autoimmune diseases (57, 189) and is known to cause several types of cancer, representing 1% of the worldwide cancer burden (187). Primary infection in children is either asymptomatic or causes mild symptoms not readily associated with EBV. In adolescents and young adults, however, EBV is most commonly recognized as the etiologic agent of acute infectious mononucleosis (230). The virus is chiefly transmitted from person to person in saliva, although it can be acquired from blood transfusions or from receipt of allogeneic donor cells or tissue. There is currently no vaccine or effective treatment for infectious mononucleosis or other EBV related diseases. Because EBV infection is limited to primates, there are no small animal models of infection except humanized mice (231). Neither humanized mice nor mice infected with the related gamma herpes virus MHV68 exhibit true infectious mononucleosis. Therefore a detailed and accurate understanding of primary infection in humans is critical for developing therapeutic tools to treat EBV related diseases.

Abundant data are available on infectious mononucleosis in humans, especially the most severe cases, as subjects are typically seen in clinic. Indeed, the antibody and cell mediated adaptive immune response to the virus, and how it wanes and changes after infectious mononucleosis presents is well established (106). However a particular knowledge gap exists regarding the events that occur between transmission and symptom onset—the incubation period—which is

unusually long, about six weeks (13, 16). In comparison, most other acute viral infections have incubation periods ranging from less than a day to a week (232). Thus in particular, we lack knowledge about initial infection events and the innate immune response to EBV in humans; although these are presumed to be critical as EBV has a multitude of innate immune evasion mechanisms (233, 234).

From in vitro studies, we know that EBV efficiently infects B cells through binding of viral gp350 and gp42 proteins to the B cell surface molecules CD21 and HLA Class II, respectively (30). However, EBV can also infect oral epithelial cells, albeit much less efficiently (23). It is unknown which cells are initially infected in the oral cavity during natural infection. One possibility is that the virus infects and replicates in oral epithelial cells early during primary infection; but infectious mononucleosis does not occur until B cells are later infected in the tonsils, and virus disseminates to the blood. The virally encoded LMP1 and LMP2 proteins are known to drive infected B cells to differentiate by acting as functional homologues of CD40 and the BCR respectively, and this also triggers migration from tonsils to the blood (235). Another model proposes that both cell types are infected early in the oral cavity, and that cycles of infection and reactivation must occur during the incubation period to ultimately produce high levels of infected B cells in circulation, which drives infectious mononucleosis (25). Alternatively, a third model is that B cells may be initially infected in tonsils where they vertically transmit virus at low levels (236). Infection would be limited to B cells in the nasopharyngeal secondary lymphoid tissue until some stochastic event caused reactivation of the virus, spreading virus to epithelial cells and

resulting in an acute increase in viral load, occurring weeks after initial infection. The dynamics of tissue tropism of EBV are interesting in this context, as the virus produced by epithelial cells is particularly efficient at infecting B cells, and vice versa (23). Each of these three models make distinct predictions about the relative levels of virus in the oral cavity versus blood during the incubation period, and would suggest different strategies to combat infection therapeutically, as antiviral compounds only target actively replicating virus.

Although it is critical to understand the viral and immunological events that occur during the incubation period, it is challenging to obtain both samples and comprehensive clinical data during this period. To address this need, we enrolled undergraduate volunteers who were naïve to EBV and monitored them routinely for natural infection, capturing timepoints within the incubation period by chance. Through frequent sampling, we were able to obtain 48 incubational samples from 40 young adult study participants. We sought to detect and quantify virus in the blood and oral cavity, with particular attention to when virus disseminates from nasopharyngeal tissue to the periphery. In addition, innate and adaptive immune responses during this period were examined, with particular emphasis on natural killer (NK) cells, plasmacytoid dendrtitic cells (pDC), CD8 T cells, and Foxp3+ T regulatory (T_{reg}) cells. Surprisingly, during the incubation period, virus was detected at low levels in peripheral blood prior to detection in the oral cavity. A dramatic reduction in blood pDC numbers and a type I interferon response were observed roughly coincident with viral increases in the blood. In contrast, the dramatic increase in CD8 T cell numbers characteristic of AIM, the distinctive

type II interferon/cell cycle gene expression signature of AIM, and changes in NK cell phenotype and T_{reg} cell numbers previously reported to occur during infectious mononucleosis were not observed until symptom onset. We discuss possible mechanisms to explain these changes and implications for the treatment of EBV related diseases.

Results

Study design

We previously described a prospective study of primary EBV infection in 66 undergraduates at the University of Minnesota (14), 59 of whom were symptomatic. As an extension of this study, we enrolled a new prospective cohort with the specific aim of more frequent sampling in order to serendipitously capture more samples from the incubation period of primary EBV infection. From both cohorts combined, a total of 48 blood and oral wash samples from 40 subjects were obtained during the historically defined incubation period (42 days). These samples were the focus of this study. Since the exact date of infection with EBV is undefinable in a study of natural infection, we designated the date of symptom onset as day "zero."

Viral dissemination into circulation occurred before large quantities of virus were detected in the oral cavity

EBV is transmitted through salivary exchange in young adults and infection is established in the oral cavity, both in squamous epithelial tissue and

lymphocytes of the Waldeyer's ring (35). Very little is known, however, about when and how EBV egresses from tonsillar tissue into the peripheral blood. In order to examine dissemination during the 6 week incubation period more closely we tested for the presence of viral genomes by both quantitative PCR (qPCR) and by a highly sensitive nested PCR. The nested PCR assay gave a much more sensitive but non-quantitative readout of viral presence, whereas the limit of detection for the quantitative PCR assay was 200 copies of EBV per milliliter of whole blood or 40 copies of virus per millilter of oral wash. Surprisingly, virus was not detected in the oral cavity by either method, until approximately a week prior to symptom onset, at which point large amounts of viral genomes were detected (Figure 3-1A). These data are expressed at a per subject level as "time to first response" in Figure 3-1C. Virus was detected in both cells from the oral wash as well as supernatant, suggesting that virus persists in the oral cavity at very low levels for the first 4-5 weeks after transmission, and then exhibits an explosive pattern of replication.

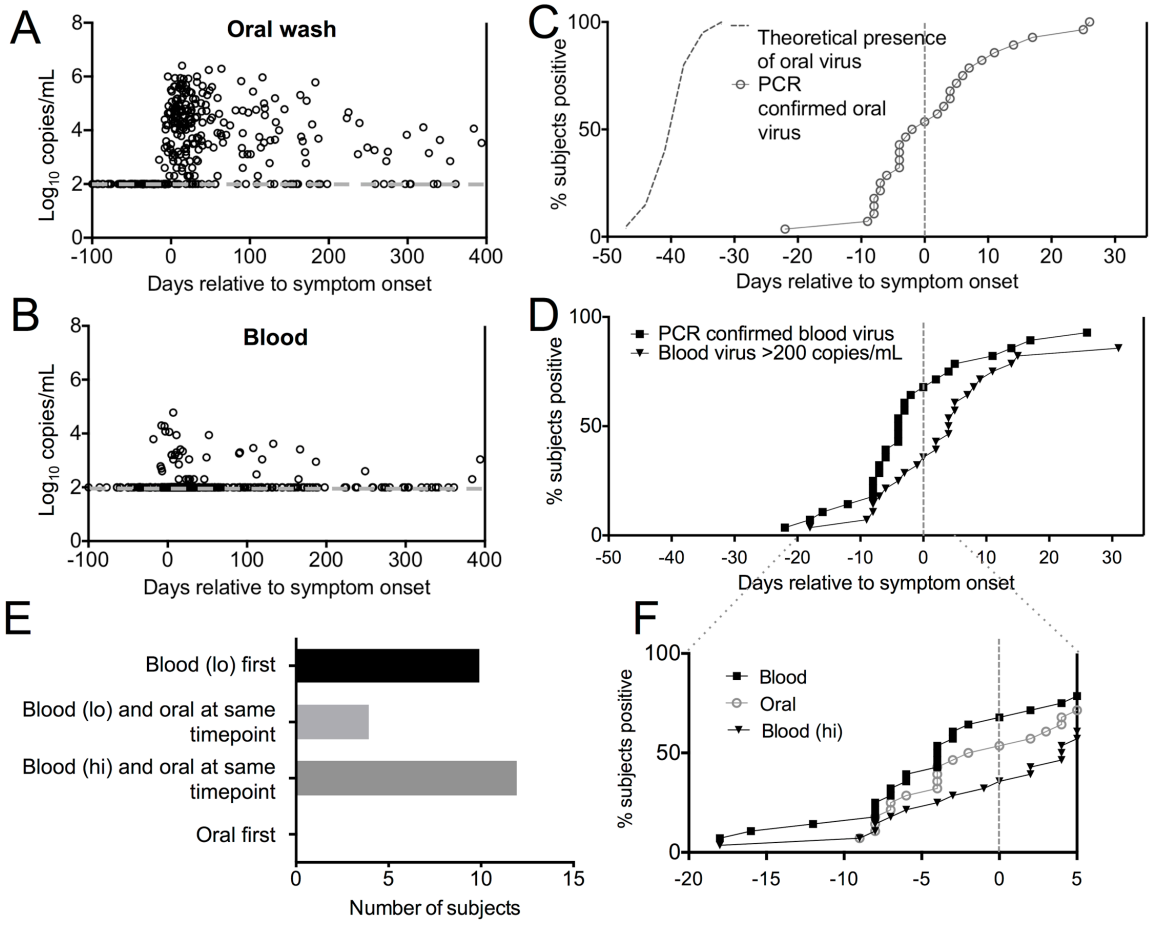


Figure 3-1. Virus detection during the incubation period. Quantitative viral load was determined by qPCR using DNA from oral wash cell pellets (A) or blood (B*).* Data are expressed as Log_{10} viral genome copies/ml of sample. The dashed gray line represents the limit of detection. (C) and (D) show the time to the first positive measurement for each subject for virus detected in the blood (D) by non-quantitative nested PCR (filled squares) or qPCR (filled inverted triangles), or in the oral cavity (C) by nested or qPCR (same results were obtained with both assays) (open circles). (E) In sequential samples from the incubation period, subjects were scored for which compartment virus was first detected in: blood (by nested PCR), blood (by qPCR), oral, or a simultaneous positive in both compartments. (F) Shows an inset comparing blood and oral cavity for the time period close to symptom onset. The results for twenty-six subjects who had a sample collected within the first two weeks of symptom onset are shown.

In contrast, virus was detected in peripheral blood as early as 22 days prior to symptom onset, but was only detected via the more sensitive nested PCR assay at this early stage (Figure 3-1 B and D). In fact, 10 subjects showed

virus detection in the blood before the oral cavity when consecutive timepoints were evaluated (Figure 3-1E). It should be noted that we were able to obtain substantially more DNA from blood cells than from oral wash cells, which could explain why low levels of virus were not detected early in the oral cavity. Nonetheless, we detected dramatically higher viral loads in the oral wash at the time of symptom onset (Figure 3-1A), suggesting efficient viral detection in oral samples. Virus detection in the blood using the less sensitive qPCR assay was delayed by at least a week (Figure 3-1F), and viral genomes >200 copies/ml were not found in any individuals until on or after timepoints where high viral loads were detected in the oral cavity (Figure 3-1E). These data provide the first description of EBV viral dynamics during the incubation period of natural infection in humans, and suggest a scenario where viral replication is self-limiting in the oral cavity for many weeks. Dissemination to the blood occurs during this “quiet period”. Closer to the time of symptom onset, virus replicates rapidly in the oral cavity, and subsequently high viral loads are detected in the blood.

Gene expression changes in peripheral blood are apparent 1-2 weeks prior to symptom onset

Previous work from our group revealed that distinct gene expression signatures were present in peripheral blood mononuclear cells early versus late after primary EBV infection (87). We sought to expand this dataset by examining the additional incubational samples obtained from our most recent cohort. Samples were evaluated by PCR SuperArray consisting of 43 genes

representative of gene changes initially observed by microarray (87). Heirarchical clustering revealed three distinct patterns from incubational samples (Figure 3-2). Subjects exhibited either no change, a type I interferon (IFN) signature, or a type II IFN/cell cycle signature. These signatures clustered temporally, segregating into these approximate time frames: (i) no change seen from -42 to -7 days prior symptom onset, (ii) a type 1 IFN signature from -15 to -3 days, and (iii) the distinctive type II IFN/cell cycle signature associated with infectious mononucleosis within days of symptom onset. Notably, the type I IFN signature was present when virus was detected only by nested PCR (low viral loads) in 3 out of 4 subjects, although 7 subjects showed no interferon response despite the presence of virus in blood by nested PCR.

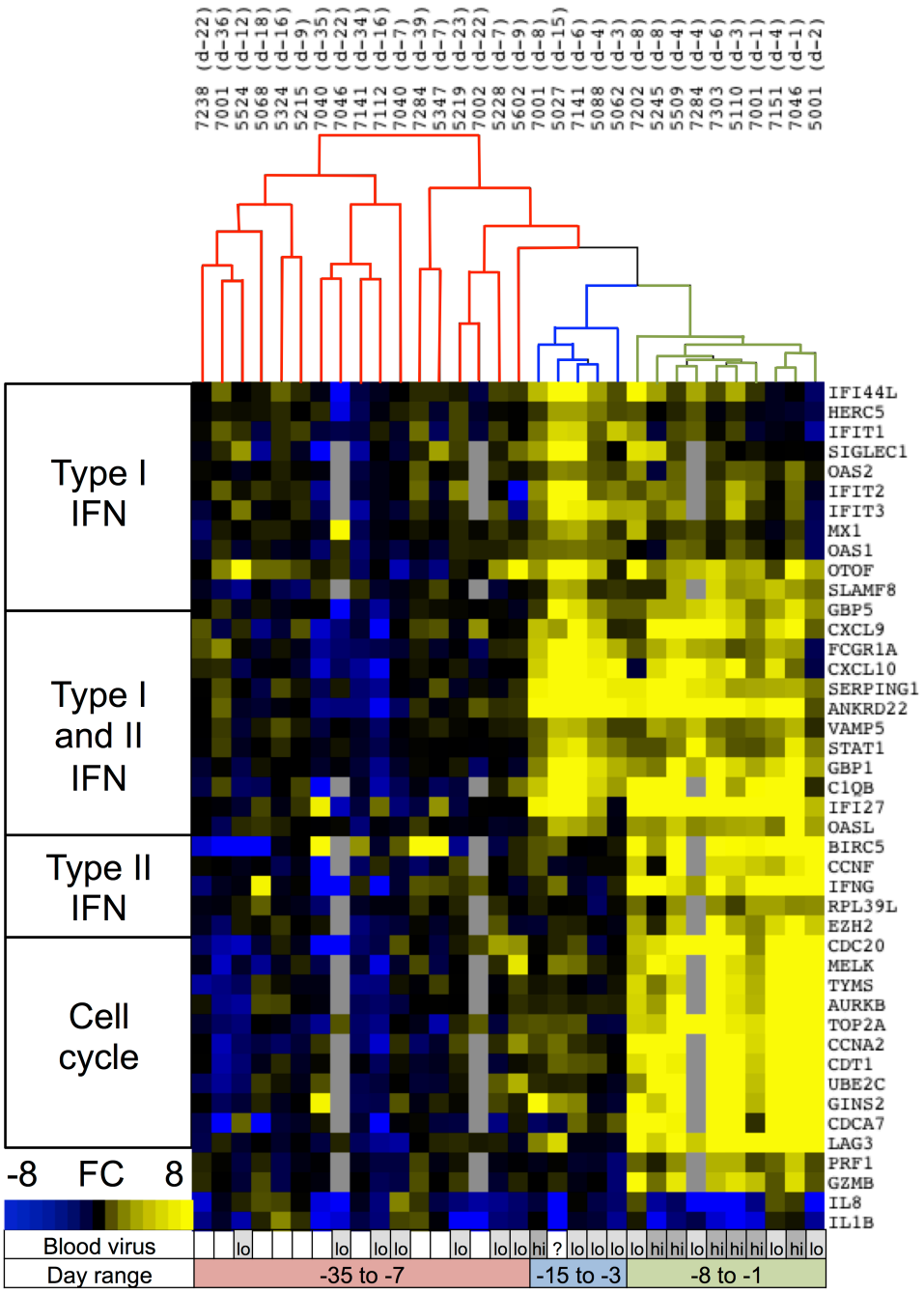


Figure 3-2. Gene expression signatures during the incubation period showed distinct kinetic patterns. 43 EBV infection signature genes were measured in total PBMC RNA. Functional categorization of the genes is shown at left. Fold change (FC) in expression was calculated compared to pre-infection samples. Heirarchical clustering (above) showed three distinct groupings: no signature (red), type I IFN (blue), and type II IFN/cell cycle (green). Subject numbers and sample collection date are indicated at top. The presence of virus by nested (lo) or qPCR (hi) is noted below, along with the range of sample days (relative to symptom onset) that each signature was observed in.

Plasmacytoid dendritic cell numbers in the circulation decrease as virus first becomes detectable

Plasmacytoid dendritic cells (pDC) are major producers of type I IFN. Although a robust type I IFN response was observed during the incubation period in some study participants, the gene expression signature was relatively transient. Khanna's group recently found pDC numbers to be reduced in acute IM patients (237). Thus, we thus sought to examine pDC numbers during the incubation period. pDC were identified as BDCA-2+ CD123+ cells amongst non-lymphoid cells (CD3, CD20, CD56 and CD14 negative) and were HLA-DR+ and CD11c- (not shown). As an example, flow plots are shown for 6 timepoints from subject 5524 (Figure 3-3A and B) Analysis of pDC from all subjects during the incubation period revealed a remarkable decline during the ten-day period leading up to symptom onset (Figure 3-3C and E). A slight decline was observable before that, but was not statistically significant. In contrast, conventional myeloid derived DC (cDC), identified as HLA-DR+ CD11c+ amongst non-lymphoid cells, were not significantly increased or decreased during either the incubation period or the early phase of infectious mononucleosis (Figure 3-3D). The loss of pDC in circulation was strongly correlated with the presence of virus in peripheral blood (Figure 3-3F). Interestingly, the reduction in pDC was as profound at timepoints where only low levels of virus were detected as when high levels of virus were detected (Figure 3-3F), and there was no significant correlation between the number of viral genomes present and the extent of pDC reduction.

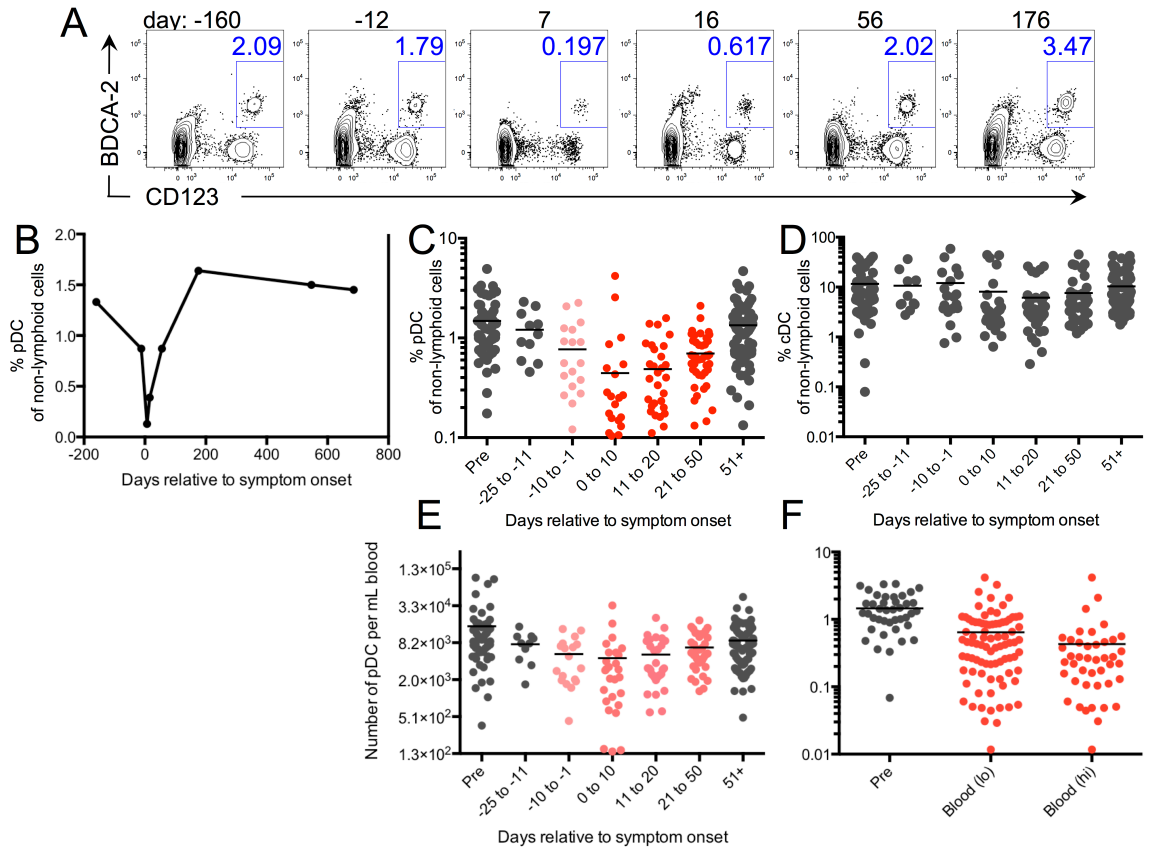


Figure 3-3. Plasmacytoid DC declined in the circulation during the incubation period and remained depressed through convalescence. (A) Representative flow cytometry plots of pDC frequencies amongst non-lymphoid cells (CD3, CD56, CD14, CD20 negative) from samples collected at multiple timepoints for one subject (5524). (B) The percentage of pDC from 5524 over time. (C) Frequencies of pDC over time are shown for all subjects. (D) Frequencies of conventional DC (cDC) ($CD11c^+$, $HLA\text{-}DR^+$ cells) are shown over time for all subjects. (E) Numbers of pDC per mL of whole blood are shown for all subjects. (F) shows the percentage of pDC in samples where virus was detected in the blood by nested PCR (Blood lo) or qPCR (Blood hi). Statistical analysis was performed using a one-way ANOVA with multiple test comparison. Light pink symbols indicate a significant difference ($p<0.05$) compared to pre-infection; darker pink symbols ($p<0.001$); red symbols ($p<0.0001$). Gray symbols indicate no statistical difference.

Natural killer cell ratios and phenotype were not affected until onset of AIM

The importance of NK cells in EBV infection has become increasingly apparent in recent years (238). Changes in NK cell phenotype during infectious

mononucleosis were reported previously, and included a loss of CD56^{bright} "immature" NK cells and a corresponding increase in CD56^{dim} "mature" NK cells (239). We also observed a reduction in CD56^{bright} NK cells in this cohort; however, unlike pDC changes, NK cell changes were not apparent until symptom onset (Figure 3-4A). We further examined a specific CD56^{dim} NKG2A+ KIR- cell subset reported to be expanded during infectious mononucleosis as a consequence of virus-induced proliferation (121). We observed a similar expansion in our cohort, but it likewise was not detected until symptom onset, and remained elevated for at least 50 days (Figure 3-4B).

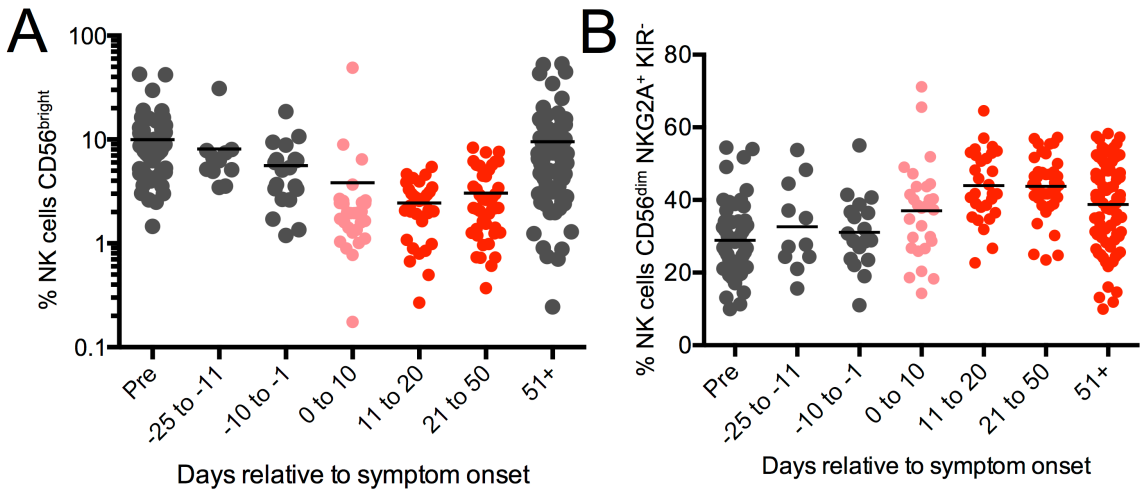


Figure 3-4. NKG2A$^+$ NK cells were expanded during AIM, but not during the incubation period. (A) Percentage of NK cells that are CD56^{bright} (immature) decreases during the first 50 days after symptom onset. (B) The percentage of NK cells that are CD56^{dim} NKG2A$^+$ KIR$^-$ increases, and remains elevated. Statistics were performed using a one-way ANOVA with multiple test comparison. Pink symbols indicate a significant difference ($p<0.05$) compared to pre-infection. Red symbols indicate a significant difference ($p<0.0001$) compared to pre-infection. Gray symbols indicate no statistical difference.

Polyclonal CD8 T cell activation was detected during the incubation period, but virus specific CD8 T cell expansion was not

CD8 T cells provide vital immune control of EBV (106), and although their expansion during infectious mononucleosis has been well documented, it is not known when they first become activated during primary infection. Using peptide:MHC I tetramers, we detected no EBV specific CD8 T cell expansion (tetramer binding cells above .05% of CD8 T cells) until timepoints near the onset of symptoms (Figure 3-5A and 3-6). Similarly, upregulation of CD11a and downregulation of CD45RA on tetramer binding T cells, indicating antigen experience, were not seen until symptom onset. The expansion of EBV specific T cells was tightly concordant with total CD8 T cell expansion, as reflected by an increased CD8:CD4 ratio (Figure 3-5 A and C). Interestingly we detected upregulation of CD38 and granzyme B on total polyclonal CD8 T cells earlier, during the incubation period (Figure 3-5A and B, and 3-6). These features of polyclonal activation correspond kinetically to when a type I IFN response was most strongly represented (Figure 3-2).

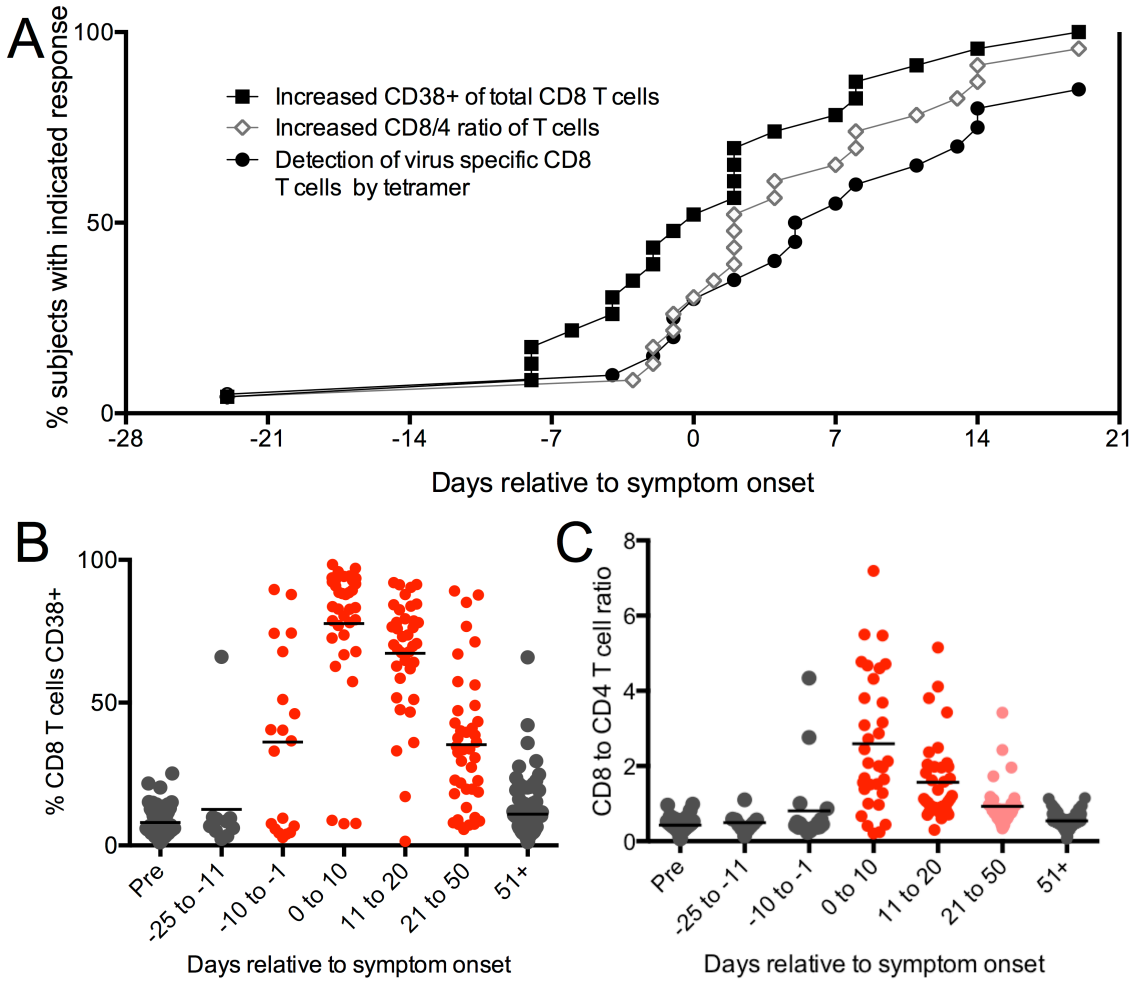


Figure 3-5. CD8 T cell activation occurred during the incubation period, although not an EBV specific response. (A), Time to first response for three distinct immune parameters is shown: CD38 upregulation on total $CD8^+$ T cells (filled squares), an increased CD8 to CD4 T cell ratio (open diamonds), or the presence of EBV tetramer binding $CD8^+$ T cells above background (0.4%) (filled circles). (B) Frequency of $CD8^+$ T cells expressing CD38 over time. (C) Ratio of $CD8^+$ to $CD4^+$ T cells over time. Statistics were performed using a one-way ANOVA with multiple test comparison. Pink symbols indicate a significant difference ($p<0.05$) compared to pre-infection. Red symbols indicate a significant difference ($p<0.0001$) compared to pre-infection. Gray symbols indicate no statistical difference.

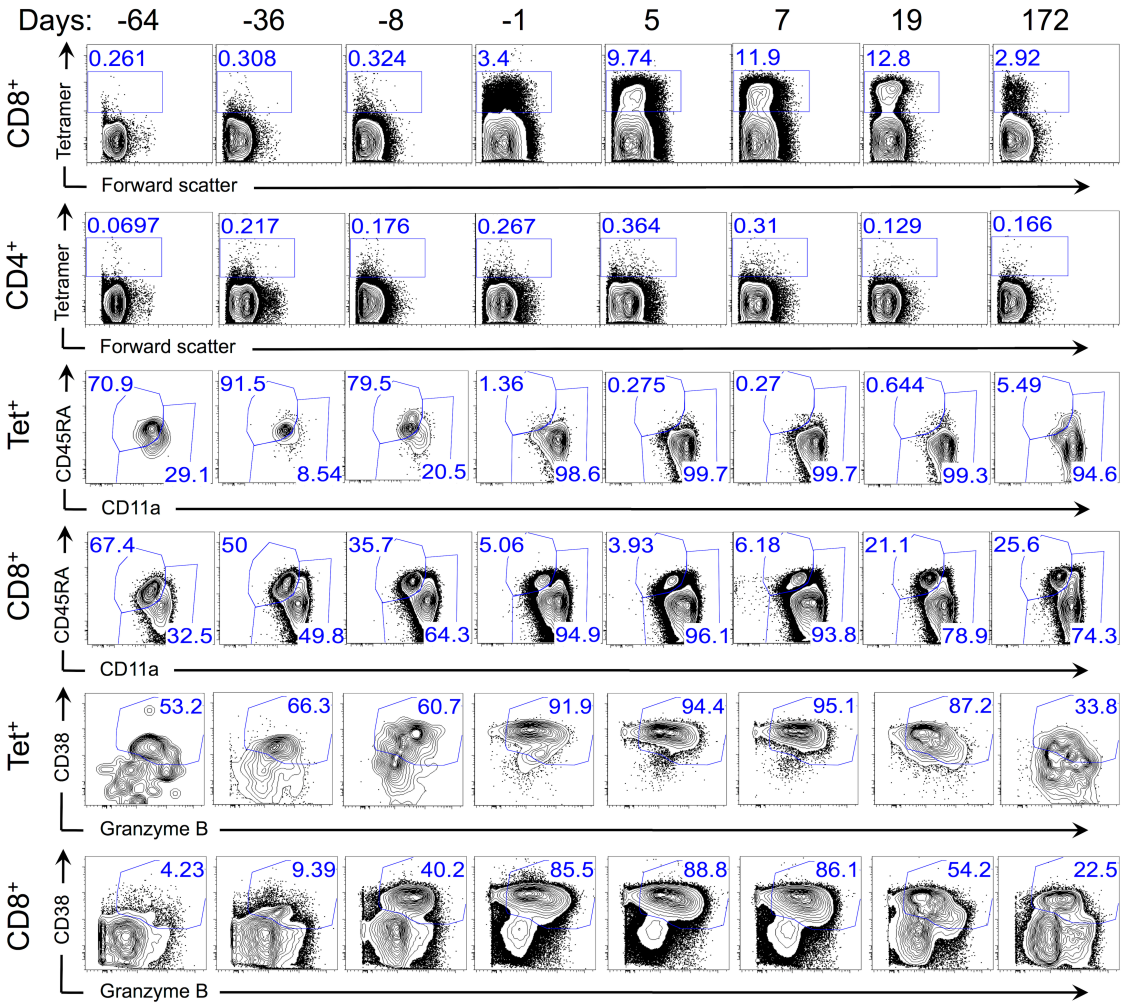


Figure 3-6. Representative flow cytometry plots of T cell analysis. The top two rows show binding of EBV tetramers (pools of 7 lytic/latent antigen/HLA tetramers prepared with APC-streptavidin) to $CD8^+$ T cells (top row) or $CD4^+$ T cells (control, second row) at the indicated time points relative to symptom onset in subject 7001. The 3rd and 5th rows show plots gated on $tetramer^+$ $CD8^+$ T cells, showing expression of memory markers (CD45RA and CD11a, 3rd row) or activation markers (CD38 and granzyme B, 5th row). The 4th and 6th rows show expression of memory (4th row) or activation markers (6th row) on total $CD8^+$ T cells.

Foxp3+ CD4 T cell numbers in the circulation decrease after presentation with acute infectious mononucleosis

Foxp3+ CD25+ T_{reg} cells, are important for the maintenance of self-tolerance and dampening chronic inflammation. A reduction in the number of

circulating $CD25^{hi}$ CD4+ T cells was previously reported in infectious mononucleosis patients (240), but it was unknown when these changes began to manifest and how long they persisted through convalescence. Analysis of individual subjects over time in our study corroborated a decrease in T_{reg} cells at the onset of infectious mononucleosis (Figure 3-7 A and B, shown for a representative subject). In all subjects, T_{reg} cells were significantly decreased only during the first ten days of infectious mononucleosis (Figure 3-7B). The overall number of CD4+ T cells, in contrast, was unchanged (Figure 3-7C). Although the fate of blood T_{reg} cells during infectious mononucleosis is unknown (e.g. whether they trafficked to tissues or died), previously reported histology of infectious mononucleosis tonsils would argue against local infiltration into tonsils, although the n of this study was very small (240).

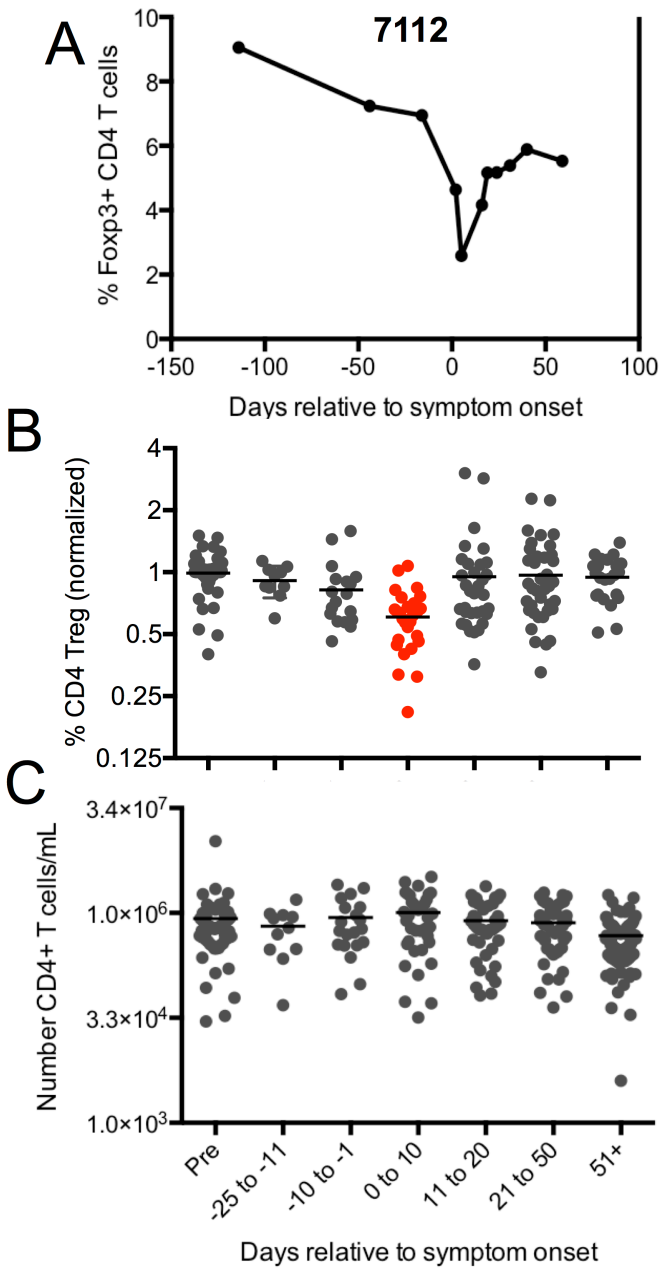


Figure 3-7. CD4$^+$ Foxp3$^+$ T cells transiently decline in the circulation at symptom onset during acute infectious mononucleosis. (A) Frequency of Foxp3$^+$CD25$^+$ cells amongst total CD4$^+$ T cells data plotted over time for a representative individual (subject 7112). (B) Normalized frequency of Foxp3$^+$ CD4 T cells over time in all subjects. Foxp3$^+$ frequencies were normalized to each subject's pre-infection baseline due to substantial variation in this population between individuals. (C) Number of CD4+ T cells per mL of whole blood over time. Statistics were performed using a one-way ANOVA with multiple test comparison. Gray values are not statistically different. Red value $p<0.0001$ compared to pre.

Discussion

Our findings have important implications regarding how EBV infection progresses through natural routes in its native host. Despite an oral transmission mode, virus was not detected in the oral cavity in appreciable quantities until subjects had presumably been infected five to six weeks. The lack of detectable virus in oral wash argues against substantial lytic replication within squamous epithelial cells early during infection. Rather, it is consistent with the idea that B cells are a major cell type initially infected in the nasopharyngeum. EBV efficiently infects B cells, particularly when virus is derived from epithelial sources, which it likely would be during transmission, since virus produced by epithelial cells packages more gp42 into virions than virus produced by B cells (30). Infected B cells are known to divide and differentiate, replicating the viral genome as an episome along with cellular division (35). This “vertical” replication would be expected to expand viral load relatively slowly, compared to active viral replication in lytically infected cells. Starting approximately 1 week before symptom onset, virus became detectable at high copy number in the oral wash. It is unclear what event precipitates this sharp increase, but it was not gradual like the decline in viral loads in the oral cavity during latency. It has been postulated that undefined signals may trigger viral reactivation in latently infected B cells (220), which could then lead to high local production of virus and large-scale infection of epithelial cells.

Interestingly, our data would also suggest that infected B cells begin to disseminate into circulation prior to events that precipitate large scale viral

production in the oral cavity. Notably, in 10 subjects we detected low levels of virus in peripheral blood at timepoints prior to detection in the oral cavity. Furthermore, in 7 subjects with low virus present in the blood, there was no type I IFN response detected. This is consistent with the idea that virus is disseminated into circulation via latently infected memory B cells (38) where it goes undetected by the innate immune system. Indeed, infected B cells were shown to transition into a “latency 0” stage that closely resembles resting memory B cells, with altered trafficking patterns (241).

Another point that emerges clearly from these data, is that systemic innate and adaptive immune responses do not occur until viral loads rise relatively late in the incubation period, either in the oral cavity or the blood. The earliest responses detected were a type I interferon response (Fig 2) and upregulation of CD38 on total CD8 T cells (Fig 5), which occurred during the 10 days prior to symptom onset. These two observations may be related, as it was previously shown that type I IFN can upregulate granzyme B in CD8 T cells, independent of activation through the antigen receptor (242). Indeed, the proportion of CD8 T cells that upregulated CD38 and Granzyme B (>80% in some individuals) at these early time points is too high to be explained by T cells recognizing virus through their antigen receptor, as clonal expansion had not been detected at these time points. Of note is the fact the type I IFN response was relatively transient and not associated with symptoms in any of the study subjects.

An adaptive immune response followed these early events, with expansion of virus specific CD8 T cells, and increased CD8:CD4 ratios rising in

all subjects in the first 10 days following symptom onset. IgM responses to EBV viral capsid antigen were also detected in this time frame. As previously reported, IgG responses to VCA developed subsequent to IgM, and IgG responses to EBNA-1 were not maximal until after 3 months. Foxp3+ T regulatory cells were reduced during symptomatic IM as reported previously (240). A similar reduction is observed in various infections in mice (243, 244), where reduction of effector cell IL-2 in the face of inflammatory cytokines was suggested to be the mechanism (245). Too little Treg activity can result in immunopathology (245), and we did observe an inverse correlation between Treg percentages during acute infection and disease severity (Spearman r= 0.4871, p=0.0251) although whether this is causative remains to be explored in EBV.

Curiously, blood NK responses were observed only after symptom onset and not earlier, although NK cells are thought to function early in infections. This result may not be entirely unexpected, as NK cells respond preferentially to lytically rather than latently infected cells (115), and our results suggest that latently infected cells are introduced into circulation prior to lytically infected cells. NK cells are thought to play a protective role in infectious mononucleosis as evidenced by NK cell depletion in humanized mice infected with EBV, which resulted in higher levels of blood viremia (115). It is possible that NK cells in the tonsil play a critical role in humans, limiting viral spread amongst epithelial cells. Furthermore, NK cells have been hypothesized to play a role in the age dependence of symptomatic primary EBV infection. For example, newborns were reported to have more than twice as many circulating $CD56^{dim}$ NKG2A+ KIR- NK

cells than adolescents (121), which could explain why children experience less EBV associated morbidity in comparison with adolescents and young adults. However, it was recently reported that infants with asymptomic primary EBV infection have blood viral loads as high as adults with infectious mononucleosis (71), which is not consistent with a model of better NK control of viremia in infants. By closely observing the viral and immune dynamics during natural infection, we offer a new hypothesis on infectious mononucleosis pathogenesis, which proposes that explosive viral replication in the oral cavity creates a situation of exaggerated CD8 T cell response that characterizes disease. It may be that children experience less infectious mononucleosis than adults despite ultimately achieving equally high levels of infected B cells in the blood because infection in the oral cavity was not initially held in check allowing the adaptive immune response to develop by the time blood levels of virus increased. Indeed, memory CD8+ T cells specific for the virus, were observed in asymptomatic infants concurrent with high viral loads (71). Ironically, it may be heightened oral innate immune surveillance in adolescents and adults, compared with children, that puts them at risk for infectious mononucleosis.

A final point of interest in our study was that circulating pDC percentages and numbers were significantly diminished during the viral incubation period. The pDC decrease initiated during the same period as a type I IFN response and polyclonal CD8 T cell activation were observed—the 10 days prior to symptom onset. Unlike the type I IFN response, which was transient, the pDC reduction was sustained for up to 50 days. The reduction was also observed at all

timepoints (except one) that showed the presence of virus, even low levels of virus, and even when a type I IFN response was not present. From this we conclude that viral infection or viral products were responsible for the pDC reduction, but it was unlikely to be mediated by the host's type I IFN response. This reduction could be related to the dynamics of pDC activation. Evidence in the literature suggests that pDC can mature or leave the circulation into tissues or secondary lymphoid organs during infections (246). Upon activation, pDC enter a maturation program that can result in progression of pDC into cDC (247); however, we did not see a corresponding increase in cDC percentages. Alternatively, reduction of circulating pDC numbers may be related to the BamHI-A rightward frame 1 (BARF1) protein secreted by EBV during lytic replication (248). BARF1 enhances viral replication and persistence in part by binding to and inhibiting the signaling of colony stimulating factor (M-CSF) (176, 179) an important factor for the survival and maintenance of pDC (178). It would be interesting to determine if primates infected with a BARF-1 deficient form of lymphocryptovirus show pDC reductions or not. The functional consequences of pDC loss from the blood during primary infection remain to be explored.

In summary, we report several novel findings about the viral and immune dynamics during the lengthy incubation period of primary EBV infection. These include relatively early dissemination of virus into circulation in a form that does not elicit immune responses. A sharp increase in viral load subsequently occurs in the oral cavity and blood within 10 days of symptom onset. An early type I IFN response during this period is associated with a marked drop in blood pDC

numbers and polyclonal CD8 T cell activation, without notable symptoms. Symptom onset coincides with a developing adaptive immune response and a strong type II interferon signature. Severity of illness correlates most strongly with increased CD8 T cell numbers, confirming the notion of infectious mononucleosis as an immunopathologic disease. The sharply increased viral loads that are presumed to drive an exuberant T cell response are already underway prior to symptom onset, providing a potential explanation for the lack of a clear-cut benefit from antiviral drugs in infectious mononucleosis (230). We also speculate that pre-existing adaptive immunity to EBV would change the dynamics of infection in the oral cavity and thereby prevent IM in adolescents and adults.

Materials and Methods

Study design

Samples analyzed here were obtained from two studies: one with a large number of subjects and less frequent sampling (Mono 5) (14) and another with a smaller number of subjects with more frequent sampling (Class of 2016). For the Mono 5 study, healthy undergraduate volunteers from the University of Minnesota were recruited in 2006 and 2007. We screened 546 participants for IgG antibodies against EBV viral capsid antigen (EBV VCA IgG) and EBV nuclear antigen-1 (EBNA-1). Of the 202 eligible EBV-naïve subjects, 143 (71%) were enrolled in the prospective study. Blood and oral washings were collected approximately every 4-8 weeks from enrolled participants during the academic year. Symptoms between visits were reported via an electronic monitoring journal. Subjects with

symptoms consistent with acute primary EBV infection were asked for an additional visit which included a physical exam, laboratory-confirmation of primary EBV infection via heterophile, EBV-specific serology, and viral titer in the oral cavity or blood. Primary EBV infection was defined as a positive EBV antibody test and the presence of EBV DNA in the blood and/or oral cavity of a subject who was previously negative for both EBV antibodies and EBV DNA. All participants were monitored with follow-up visits. The Class of 2016 study was similar to the above except blood and/or oral washings were collected approximately every 2 weeks. We screened 279 participants and 87 EBV-naïve subjects were enrolled in 2012, 16 of whom experienced primary EBV infection during their 9 month freshman year.

Ethics Statement

All participants gave written informed consent and the University of Minnesota Institutional Review Board approved all protocols used.

Samples collection and handling

Subjects gave oral wash samples by gargling with 22mL of normal saline. Peripheral blood was obtained via venipuncture into EDTA Vacutainer® tubes (Fisher Scientific). Blood Peripheral blood mononuclear cells (PBMCs) were isolated by Accuspin™ System-Histopaque®-1077 (Sigma-Aldrich) density gradient centrifugation per manufacturer's instructions. PBMC were divided into 1x10^7 cells/mL aliquots in a 90% FBS and 10% dimethylsulfoxide solution to

prevent cell damage (Sigma-Aldrich). Vials were placed inside Mr. Frosty freezing containers (Thermo Scientific) and frozen at -80°C per the manufacturer's instructions, then transferred to liquid nitrogen for long term storage. 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, RNA processing, and/or DNA processing.

Flow cytometry staining and analysis

Multiple time points were chosen from subjects who gave a blood sample during the incubation period. 1 to 2 x10^6 PBMCs from each of these time points were used in each stain. The following antibodies were used to identify relevant surface and intracellular markers: CD3 (UCHT1), CD4 (RPA-T4), CD11a (HI111), CD20 (2H7), CD123 (6H6), PD-1 (MIH4), CD25 (BC96), Foxp3 (PCH101) (eBioscience); CD45RA (HI100), CD38 (HIT2), CD16 (3G8), CD57 (HCD57), CD14 (M5E2), CD19 (HIB19), BDCA-2 (201A) (BioLegend); CD56 (NCAM16.2), HLA-DR (G46-6), CD11c (B-ly6) (Becton Dickinson); NKG2A (Z199), KIR2DL1/2DS1 (EB6B), KIR2DL2/2DL3/2DS2 (GL183) (Beckman Coulter); CD8 (3B5) (Invitrogen); KIR3DL1 (DX9), KIR3DL2 (539304) (R&D systems). Intracellular granzyme B staining was performed using the Cytofix/Cytoperm kit

per the manufacturer's instructions (BD). Intranuclear Foxp3 staining was performed using the Foxp3 / Transcription Factor Staining Buffer Set per the manufacturer's instructions (eBioscience). All samples were acquired using an LSR II (BD) and analyzed with FlowJo software (TreeStar).

RNA and DNA extraction

For each sample, 1-2 x 10^6 PMBCs were used for each RNA extraction. Cells were first homogenized using QIAshredder columns (Qiagen) per the manufacturer's instructions. RNA was then extracted using RNeasy kit (Qiagen) with on-column DNase step (Qiagen) per the manufacturer's instructions. RNA was then quantified using a Nanodrop 2000/2000c spectrophotometer (Thermo Scientific) and kept frozen at -80°C. DNA extractions were performed with the Qiagen QIAmp Blood Mini kit per the manufacture's instructions, using either 200 µL of whole blood or 5x10^6 PBMC.

Peptide MHC class I tetramers reagents

An EBV BMLF1259–267 (GLCTLVAML)-A*0201 tetramer reagent was purchased from ProImmun. Other biotinylated MHC-peptide monomers were obtained from the National Institutes of Health (NIH) tetramer facility: EBV BRLF1109–117 (YVLDHLIVV)-A*0201, EBV BRLF1147–155 (RVRAYTYSK)-A*03, EBV BZLF1190–197 (RAKFKQLL)-B*08, EBV EBNA3A325–333 (FLRGRAYGL)-B*08, EBNA3A379–387 (RPPIFIRRL)-B*07, and EBNA3A603–611 (RLRAEAQVK)-A*03. Before use, APC-streptavidin (Invitrogen) was added

to monomers at a 4:1 molar ratio overnight in the dark at 4°C to generate fluorescent pMHCI tetramer complexes. All tetramers were stored in the dark at 4°C.

cDNA synthesis

cDNA was generated with 100ng of starting RNA using the SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen) per the manufacturer's instructions. Samples were stored at -20°C.

Quantitative PCR

Standard quantitative PCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche) per the manufacture's instructions. Additional data were generated with precoated "SuperArray" PCR plates. 43 genes were selected for analysis by PCR from a larger list of changed genes in IM subjects and other acute viral infections that comprised relevant functional groupings as assessed by Ingenuity Pathway Analysis as previously described (87). 384-well SuperArray plates pre-coated with primers for the desired 43 genes plus 5 controls were obtained as a custom order from SABiosciences. 10 µL of cDNA per subject was used with RT2 Real-time SYBR green/Rox PCR master mix (SABiosciences) for qRT-PCR analysis. Products were detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The genes *ACTB*, *B2M*, and *RPL13A* were used as housekeeping genes during the calculation of fold changes. Fold changes were calculated as: 2^(Δ Acute Housekeeping Control –

Baseline Housekeeping Control)/2^(Δ Acute Gene of Interest – Baseline Gene of Interest).

Heatmap generation

Fold change values obtained by quantitative PCR were imported into the open source program Cluster 3.0, (228) which clustered the genes hierarchically using a Pearson non-averaged correlation and average linkage. Heatmaps were then visualized using the program Java Treeview. (229)

Nested PCR

PCR was performed with the HotStarTaq master mix kit (Qiagen) per the manufacture's instructions. Primers specific for *EBNA1* (249) were used (outer-F 5'-GTA GAA GGC CAT TTT TCC AC-3'; outer-R 5'-CTC CAT CGT CAA AGC TGC A-3'; inner-F 5'-AGA TGA CCC AGG AGA AGG CCC AAG C-3'; inner-R 5'-CAA AGG GGA GAC GAC TCA ATG GTG T-5').

Chapter 4

Discussion

DISCUSSION

Most infected individuals carry EBV for life without significant disease, and yet EBV continues to be a significant human pathogen. Despite over fifty years of EBV related research it is still unclear why some people experience infectious mononucleosis and why a small portion of the infected population progress to EBV related sequelae. Many unknowns remain regarding the progression of primary EBV infection, particularly in regard to how immunologic controls influence outcomes during the acute and chronic phases of infection. While our analysis has largely been limited to the acute phase of infection, examination of events surrounding primary infection may inform important aspects of future treatment regimens or vaccine formulations.

Specifically, our results seem to support the notion that infectious mononucleosis in an immunopathologic disease, and is not directly driven by EBV. This stance remains somewhat controversial given previous work from our group (14) and one other study which examined severity of illness (15). A correlation was observed between high viral loads and severity of illness in these studies, though whether or not viremia is truly driving disease or is merely a passenger or mechanistically unrelated. A stronger correlation was observed between CD8 T cell numbers and severity of illness in the study where cell phenotyping was performed (14). Evidence from individuals with asymptomatic seroconversion (71) correlates more closely with the data collected in the studies summarized in this thesis. Taken together, we propose that high viral loads are not necessarily indicative of symptomatic illness, and that overexpansion of CD8

T cells is more likely to cause infectious mononucleosis. This is consistent with our gene expression profiling studies which found a higher similarity between infectious mononucleosis and hemophagocytic diseases than between infectious mononucleosis acute viral infections (87). Furthermore, EBV is known to cause severe immunopathologic complications in certain cases (84, 134). This may provide an explanation as to why antiviral drugs have comparatively little efficacy during infectious mononucleosis as compared with other acute herpesvirus reactivations.

As an adjunct to these considerations, viral infection of peripheral B cells in the periphery and those in the oropharynx appear to be controlled independently. We observed a disconnect between viral dissemination from the oral cavity and viral shedding in the saliva. Our data do not adequately explain why viral shedding in the oral cavity should happen so suddenly after presumably being present in tonsillar tissues for several weeks. In many individuals in our study, large-scale replication of EBV in the oral cavity (likely in squamous epithelial cells given the envelope glycoprotein content of salivary EBV) was observed after high sensitivity detection of virus in the blood. While a larger number of oral cells would likely surmount this sensitivity issue, the rapidly increasing number of copies of EBV is still inconsistent with even an exponential mode of replication. Thus, some other control is in place to limit viral replication in the oral cavity during primary infection.

It seems probable that this control is mediated by either innate immune mechanisms local to the oropharynx, specifically through type I IFN, or by factors

intrinsic to the virus itself. CD8 T cell infiltration into the tonsil remains relatively poor, even during the acute phase of infectious mononucleosis when EBV specific CD8 T cells make up a large proportion of circulating lymphocytes (250). Furthermore, T cells that do infiltrate tend to be specific for latent rather than lytic antigens (250). NK cells are unlikely to be involved during the incubation period as they preferentially target only lytically infected cells (113). No EBV specific response is detectable until the onset of symptoms, either in terms of expansion or activation. These findings support a model in which oral cells may be only rarely infected, and the largest reservoir of virus in the oral cavity may be transformed B cells. These Latency III cells replicate as lymphoblastoid cells for the first several weeks, simultaneously "vertically" replicating the EBV episome. Some triggering reactivation event precipitates a switch from Latency III to lytic infection, allowing for infection of epithelial cells by newly budded B cell derived virus.

Controlling early infection in epithelial cells may therefore not be of tremendous import when formulating an effective prophylactic vaccine. Indeed, what is the goal of an effective vaccine? Complete prevention of viral latency through sterilizing immunity has long been the gold standard objective of most prophylactic vaccines, especially for other oncogenic viruses such as hepatitis and human papilloma virus. Previous work on the development of vaccines for HHVs however, has shown that it may not be necessary for preventing disease. The vaccine for varicella zoster virus has been considered successful although it does not provide sterilizing immunity against infection with wild-type virus (251).

Given hypotheses regarding high viral titres being related to EBV related disease, simply reducing the viral setpoint may be sufficient to abrogate future sequelae and complications.

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Appendix

Gene Symbol	Common and/or Official Name	Fold Change
IFI27	interferon, alpha-inducible protein 27	10.416
TYMS	TS or thymidylate synthetase	8.973
KIAA0101	PAF, PAF15, p15PAF, or p15(PAF)	8.162
CDC20	cell division cycle 20 homolog	7.391
CDCA7	JPO1 or cell division cycle associated 7	6.864
TOP2A	topoisomerase (DNA) II alpha	6.692
ANKRD22	ankyrin repeat domain 22	6.680
CCNB2	cyclin B2	6.644
MELK	maternal embryonic leucine zipper kinase	6.491
CCNA2	cyclin A2	6.451
GINS2	GINS complex subunit 2	6.413
GBP1	guanylate binding protein 1, interferon-inducible	6.396
CDC45L	cell division cycle 45 homolog	6.373
CDCA5	SORORIN or cell division cycle associated 5	6.267
LAG3	lymphocyte-activation gene 3	6.069
MT1E	metallothionein 1E	6.049
IFNG	interferon, gamma	5.876
UBE2C	ubiquitin-conjugating enzyme E2C	5.794
TK1	thymidine kinase 1, soluble	5.669
KIFC1	kinesin family member C1	5.554
BIRC5	baculoviral IAP repeat containing 5	5.553
MCM4	minichromosome maintenance complex component 4	5.537
CEP55	centrosomal protein 55kDa	5.532
GBP5	guanylate binding protein 5	5.441
CDT1	chromatin licensing and DNA replication factor 1	5.278
AURKB	aurora kinase B	5.263
CDC2	CDK1 or cyclin-dependent kinase 1	5.246
MCM2	minichromosome maintenance complex component 2	5.225
CDKN3	cyclin-dependent kinase inhibitor 3 or KAP1	5.170
DLG7	HURP or DLGAP5	5.162
HMMR	RHAMM or hyaluronan-mediated motility receptor	5.047
ACOT7	BACH or acyl-CoA thioesterase 7	5.018
NCAPG	non-SMC condensin I complex, subunit G	4.972
NUSAP1	nucleolar and spindle associated protein 1	4.965
CHEK1	checkpoint kinase 1 or CHK1	4.819
MCM10	minichromosome maintenance complex component 10	4.812
OIP5	LINT-25	4.774
CENPM	centromere protein M	4.772
TIMD4	TIM4 or T-cell immunoglobulin and mucin domain containing 4	4.765
PTTG1	pituitary tumor-transforming 1	4.720
KIF11	EG5	4.661
ASPM	asp (abnormal spindle) homolog, microcephaly	4.653

	associated	
KIF20A	MKLP2	4.584
CXCL10	IP-10	4.529
PTTG3	PTTG3P or pituitary tumor-transforming 3, pseudogene	4.434
STMN1	stathmin 1	4.222
FEN1	flap structure-specific endonuclease 1	4.196
POLE2	polymerase (DNA directed), epsilon 2, accessory subunit	4.169
TRIP13	thyroid hormone receptor interactor 13	4.130
TTK	MPS1	4.075
STAT1	signal transducer and activator of transcription 1	4.036
CENPN	centromere protein N	3.982
OASL	2'-5'-oligoadenylate synthetase-like	3.975
TPX2	microtubule-associated, homolog	3.975
CENPA	centromere protein A	3.963
GMNN	geminin, DNA replication inhibitor	3.918
PLK4	polo-like kinase 4 or SAK	3.909
SLC27A2	solute carrier family 27 (fatty acid transporter), member 2	3.862
POLQ	polymerase (DNA directed), theta	3.840
CKAP2L	cytoskeleton associated protein 2-like	3.833
TUBA1C	tubulin, alpha 1c	3.825
CD38	CD38 molecule	3.823
MT2A	metallothionein 2A	3.823
EXO1	exonuclease 1	3.814
RAD51AP1	RAD51 associated protein 1	3.807
GBP4	guanylate binding protein 4	3.796
BUB1	budding uninhibited by benzimidazoles 1	3.754
STIL	SCL/TAL1 interrupting locus or SIL	3.721
PCNA	proliferating cell nuclear antigen	3.675
HJURP	Holliday junction recognition protein	3.671
AURKA	aurora A or aurora kinase A	3.669
KIF14	kinesin family member 14	3.659
CD8A	CD8a molecule	3.632
APOBEC3H	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3H	3.607
MND1	meiotic nuclear divisions 1 homolog	3.602
CDCA8	BOREALIN or cell division cycle associated 8	3.596
CKS2	CDC28 protein kinase regulatory subunit 2	3.592
PRC1	protein regulator of cytokinesis 1	3.574
MCM6	minichromosome maintenance complex component 6	3.566
CCNF	cyclin F or FBX1	3.553
SPC24	NDC80 kinetochore complex component, homolog	3.417
E2F2	E2F transcription factor 2	3.413
KIF15	HKLP2	3.413
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	3.392

C12orf48	PARPBP; PARI	3.391
KIF2C	MCAK	3.390
C18orf56	chromosome 18 open reading frame 56	3.389
GZMH	granzyme H	3.318
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	3.286
CDCA2	Repo-Man or cell division cycle associated 2	3.242
FANCI	Fanconi anemia, complementation group I	3.234
ATAD2	ANCCA or ATPase family, AAA domain containing 2	3.232
UBE2T	ubiquitin-conjugating enzyme E2T	3.214
C1QB	complement component 1, q subcomponent, B chain	3.208
LMNB1	lamin B1	3.168
CDCA3	cell division cycle associated 3	3.160
ASF1B	ASF1 anti-silencing function 1 homolog B or CIA-II	3.147
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	3.146
PHGDH	phosphoglycerate dehydrogenase	3.141
RFC4	replication factor C (activator 1) 4	3.111
WDR51A	POC1A or PIX2	3.107
MT1G	metallothionein 1G	3.048
PSAT1	phosphoserine aminotransferase 1	3.040
CHI3L2	YKL-39 or chitinase 3-like 2	3.026
C6orf173	CENPW; CUG2; centromere protein W	2.999
NDC80	HEC1; TID3; KNTC2	2.978
MT1A	metallothionein 1A	2.950
C3orf14	chromosome 3 open reading frame 14	2.937
IL32	interleukin 32	2.909
VAMP5	vesicle-associated membrane protein 5	2.907
MCM5	minichromosome maintenance complex component 5	2.905
MTE	MT1IP; metallothionein 1I, pseudogene	2.897
FCGR1B	Fc fragment of IgG, high affinity Ib, receptor (CD64)	2.876
CHAF1B	CAF1; chromatin assembly factor 1, subunit B (p60)	2.853
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	2.844
HELLS	LSH; helicase, lymphoid-specific	2.837
RRM1	ribonucleotide reductase M1	2.834
RACGAP1	MgcRacGAP; Rac GTPase activating protein 1	2.814
SCD	SCD1; stearoyl-CoA desaturase (delta-9-desaturase)	2.781
PSME2	PA28B; proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	2.769
CDC7	CDC7L1; cell division cycle 7 homolog (S. cerevisiae)	2.767
C16orf75	RMI2, RecQ mediated genome instability 2, homolog (S. cerevisiae)	2.747
RAD51	RAD51 homolog (S. cerevisiae)	2.747
SMC2	CAP-E; structural maintenance of chromosomes 2	2.738

FHL2	four and a half LIM domains 2	2.724
SMC4	CAP-C; structural maintenance of chromosomes 4	2.692
LY6E	RIGE; SCA2; TSA-1	2.682
RBBP8	CTIP; SCKL2; retinoblastoma binding protein 8	2.682
EPSTI1	BRESI1; epithelial stromal interaction 1 (breast)	2.664
EZH2	enhancer of zeste homolog 2 (Drosophila)	2.651
RPL29	HIP; L29; ribosomal protein L29	2.651
HMGA1	high mobility group AT-hook 1	2.636
CD8B	CD8b molecule	2.630
C20orf100	TOX2; TOX high mobility group box family member 2	2.628
ENDOGL1	EXOG; endo/exonuclease (5'-3'), endonuclease G-like	2.618
TIMELESS	TIM; timeless homolog (Drosophila)	2.618
C18orf24	SKA1; spindle and kinetochore associated complex subunit 1	2.610
FBXO6	FBS2; F-box protein 6	2.601
MYL6B	myosin, light chain 6B, alkali, smooth muscle and non-muscle	2.588
USP54	ubiquitin specific peptidase 54	2.587
FLJ33590	CXXC11; CXXC finger protein 11	2.583
PYHIN1	IFIX; pyrin and HIN domain family, member 1	2.564
RARRES3	RIG1; TIG3; retinoic acid receptor responder (tazarotene induced) 3	2.560
LAP3	leucine aminopeptidase 3	2.543
TROAP	TASTIN; trophinin associated protein	2.543
GFI1	SCN2; growth factor independent 1 transcription repressor	2.540
PSMB8	LMP7; proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	2.524
MCM7	minichromosome maintenance complex component 7	2.522
GCH1	GTP cyclohydrolase 1	2.521
TUBG1	tubulin, gamma 1	2.516
GALM	galactose mutarotase (aldose 1-epimerase)	2.500
DONSON	CIITA; downstream neighbor of SON	2.487
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	2.487
MLF1IP	CENPU; MLF1 interacting protein	2.486
C17orf53	chromosome 17 open reading frame 53	2.485
GINS3	PSF3; GINS complex subunit 3 (Psf3 homolog)	2.485
IFI6	G1P3; interferon, alpha-inducible protein 6	2.483
C16orf59	chromosome 16 open reading frame 59	2.480
GBP2	guanylate binding protein 2, interferon-inducible	2.476
NME1	NM23; NM23-H1; NME/NM23 nucleoside diphosphate kinase 1	2.464
KLRD1	CD94; killer cell lectin-like receptor subfamily D, member 1	2.460

CDK2	cyclin-dependent kinase 2	2.457
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	2.452
PRR11	proline rich 11	2.447
PSMA3	HC8; proteasome (prosome, macropain) subunit, alpha type, 3	2.447
OBFC2B	NABP2; SSB1; OBFC2B; nucleic acid binding protein 2	2.443
C1orf41	HSPB11; heat shock protein family B (small), member 11	2.437
OTOF	DFNB9; DFNB6; otoferlin	2.437
RPL39L	ribosomal protein L39-like	2.436
UBE2L6	UBCH8; ubiquitin-conjugating enzyme E2L 6	2.436
MCM3	minichromosome maintenance complex component 3	2.433
ADA	adenosine deaminase	2.429
CACYBP	SIP; calcyclin binding protein	2.429
C6orf190	THEMIS; thymocyte selection associated	2.428
CKS1B	CKS1; CDC28 protein kinase regulatory subunit 1B	2.427
SLAMF8	SLAM family member 8	2.420
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2.414
ZWILCH	Zwilch, kinetochore associated, homolog (Drosophila)	2.412
GTSE1	G-2 and S-phase expressed 1	2.409
NUDT1	MTH1; nudix (nucleoside diphosphate linked moiety X)-type motif 1	2.408
ZNF683	zinc finger protein 683	2.396
PPIL5	LRR1; leucine rich repeat protein 1	2.387
STK39	SPAK; serine threonine kinase 39	2.377
WARS	tryptophanyl-tRNA synthetase	2.376
TFDP1	transcription factor Dp-1	2.369
PAICS	ADE2; AIRC; PAIS; ADE2H1	2.362
ANLN	anillin, actin binding protein	2.359
DUT	dUTPase; deoxyuridine triphosphatase	2.356
PDCD1	PD-1; programmed cell death 1	2.344
C6orf129	CCDC167; coiled-coil domain containing 167	2.343
PRF1	perforin 1 (pore forming protein)	2.335
CASP7	caspase 7, apoptosis-related cysteine peptidase	2.330
IFI16	PYHIN2; interferon, gamma-inducible protein 16	2.324
CCNE1	cyclin E1	2.323
PSMB2	proteasome (prosome, macropain) subunit, beta type, 2	2.323
CCDC28B	coiled-coil domain containing 28B	2.318
FGFBP2	fibroblast growth factor binding protein 2	2.313
HMGB2	high mobility group box 2	2.313
BRCA1	breast cancer 1, early onset	2.308
GCUD2	FAM72D; family with sequence similarity 72, member D	2.299
TIPIN	TIMELESS interacting protein	2.293

C16orf33	SNRNP25; small nuclear ribonucleoprotein 25kDa (U11/U12)	2.287
WDR34	WD repeat domain 34	2.287
TUBB	tubulin, beta class I	2.286
RTP4	IFRG28; receptor (chemosensory) transporter protein 4	2.275
SLAMF7	CS1; CD319; CRACC	2.273
MCOLN2	TRPML2; mucolipin 2	2.266
CCDC99	SPDL1; spindle apparatus coiled-coil protein 1	2.262
CCDC34	coiled-coil domain containing 34	2.261
FCRL6	Fc receptor-like 6	2.261
PARP9	BAL1; poly (ADP-ribose) polymerase family, member 9	2.260
RFC5	RFC36; replication factor C (activator 1) 5, 36.5kDa	2.259
PSMG1	PAC1; DSCR2; proteasome (prosome, macropain) assembly chaperone 1	2.252
C1orf135	AUNIP; AIBP; aurora kinase A and ninein interacting protein	2.251
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	2.249
ORC1L	ORC1; PARC1; origin recognition complex, subunit 1	2.247
PHF19	PCL3; PHD finger protein 19	2.247
C9orf140	SAPCD2; suppressor APC domain containing 2	2.243
CENPL	centromere protein L	2.239
NUF2	CDCA1; NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	2.232
IL28RA	interleukin 28 receptor, alpha (interferon, lambda receptor)	2.222
PXMP2	PMP22; peroxisomal membrane protein 2, 22kDa	2.218
RANBP1	RAN binding protein 1	2.218
FASLG	FASL; CD178; CD95L; TNFSF6	2.216
EBP	emopamil binding protein	2.210
PGAM1	phosphoglycerate mutase 1 (brain)	2.209
ALS2CR4	TMEM237	2.206
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	2.205
MRPL10	mitochondrial ribosomal protein L10	2.200
SH2D1A	LYP; SAP; XLP; SH2 domain containing 1A	2.199
IFIT3	IFIT4; RIG-G; interferon-induced protein with tetratricopeptide repeats 3	2.197
ARPC5L	ctin related protein 2/3 complex, subunit 5-like	2.196
TIPRL	TIP41, TOR signaling pathway regulator-like (S. cerevisiae)	2.194
POLA2	polymerase (DNA directed), alpha 2, accessory subunit	2.187
UNG	UNG1; UNG2; uracil-DNA glycosylase	2.186
LGALS3BP	MAC-2-BP; G3BP; lectin, galactoside-binding, soluble, 3 binding protein	2.184

RPA3	replication protein A3, 14kDa	2.177
SNRPA	U1A; small nuclear ribonucleoprotein polypeptide A	2.177
BAK1	BAK; BCL2-antagonist/killer 1	2.176
APOL3	apolipoprotein L, 3	2.173
TCTEX1D2	Tctex1 domain containing 2	2.173
NCAPD2	CNAP1; non-SMC condensin I complex, subunit D2	2.168
GZMB	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	2.167
APITD1	MHF1; CENPS	2.165
RPS7	S7; ribosomal protein S7	2.165
FAM33A	SKA2; spindle and kinetochore associated complex subunit 2	2.164
AGK	MULK; acylglycerol kinase	2.156
PTPLAD1	B-IND1; protein tyrosine phosphatase-like A domain containing 1	2.154
MRPL12	mitochondrial ribosomal protein L12	2.153
NUP37	nucleoporin 37kDa	2.153
PRIM1	primase, DNA, polypeptide 1 (49kDa)	2.145
DNAJC9	DnaJ (Hsp40) homolog, subfamily C, member 9	2.134
CENPK	centromere protein K	2.131
MTHFD1	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase	2.131
KIF19	kinesin family member 19	2.129
CXCR3	MigR; IP10-R; chemokine (C-X-C motif) receptor 3	2.128
RAB33A	RabS10; RAB33A, member RAS oncogene family	2.124
FANCG	Fanconi anemia, complementation group G	2.123
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	2.122
CXCL9	MIG; chemokine (C-X-C motif) ligand 9	2.120
ISG15	ISG15 ubiquitin-like modifier	2.115
XTP3TPA	DCTPP1; DCTPP1 dCTP pyrophosphatase 1	2.115
MT1X	metallothionein 1X	2.111
PIF1	PIF1 5'-to-3' DNA helicase homolog (S. cerevisiae)	2.109
CDC42	cell division cycle 42 (GTP binding protein, 25kDa)	2.107
SAMD3	sterile alpha motif domain containing 3	2.102
TRMT5	TRM5; tRNA methyltransferase 5 homolog (S. cerevisiae)	2.101
KEAP1	kelch-like ECH-associated protein 1	2.099
PTPN7	HEPTP; protein tyrosine phosphatase, non-receptor type 7	2.099
HMGN2	high mobility group nucleosomal binding domain 2	2.087
GART	phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, phosphoribosylaminoimidazole synthetase	2.082
PAQR4	progestin and adipoQ receptor family member IV	2.078
PSMA5	PSC5; proteasome (prosome, macropain) subunit, alpha type, 5	2.078
RALY	RNA binding protein, autoantigenic (hnRNP-	2.078

	associated with lethal yellow homolog (mouse))	
RAD54L	hRAD54; RAD54-like (S. cerevisiae)	2.077
TGFBR3	betaglycan; transforming growth factor, beta receptor III	2.076
SLBP	stem-loop binding protein	2.071
KCNK10	TREK-2; potassium channel, subfamily K, member 10	2.070
MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	2.065
RFC3	RFC38; replication factor C (activator 1) 3, 38kDa	2.065
MSH6	mutS homolog 6 (E. coli)	2.063
LIMA1	EPLIN; LIM domain and actin binding 1	2.062
UCK2	UK; uridine-cytidine kinase 2	2.059
GINS4	GINS complex subunit 4 (Sld5 homolog)	2.058
DDX39	URH49; DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A	2.056
EXOSC3	exosome component 3	2.056
C1orf112	chromosome 1 open reading frame 112	2.047
POLA1	polymerase (DNA directed), alpha 1, catalytic subunit	2.044
SPON2	spondin 2, extracellular matrix protein	2.044
CASP3	caspase 3, apoptosis-related cysteine peptidase	2.043
MT1F	metallothionein 1F	2.040
DPP3	DPPIII; dipeptidyl-peptidase 3	2.039
CBX5	HP1; HP1A; chromobox homolog 5	2.038
FBXO5	F-box protein 5	2.036
TXNL2	GLRX3; PICOT	2.036
BLM	Bloom syndrome, RecQ helicase-like	2.034
INCENP	inner centromere protein antigens 135/155kDa	2.034
DCLRE1A	SNM1; SNM1A; DNA cross-link repair 1A	2.031
IFI35	IFP35; interferon-induced protein 35	2.031
CTPS	CTPS1; CTP synthase 1	2.030
DSN1	KNL3; MIS13; DSN1, MIND kinetochore complex component, homolog (S. cerevisiae)	2.030
MRPL22	mitochondrial ribosomal protein L22	2.026
PPP1CA	PP-1A; protein phosphatase 1, catalytic subunit, alpha isozyme	2.022
CDC123	cell division cycle 123 homolog (S. cerevisiae)	2.019
VDAC1	voltage-dependent anion channel 1	2.018
PSMA4	HC9; PSC9; proteasome (prosome, macropain) subunit, alpha type, 4	2.015
C7orf24	GGCT; CRF21; gamma-glutamylcyclotransferase	2.009
KNTC1	ROD; kinetochore associated 1	2.009
EIF2B2	eukaryotic translation initiation factor 2B, subunit 2 beta, 39kDa	2.008
MRPL39	mitochondrial ribosomal protein L39	2.008
SIRPG	signal-regulatory protein gamma	2.008
TUBB2C	tubulin, beta 4B class IVb	2.008
TMEM97	MAC30; transmembrane protein 97	2.007

ACTL6A	BAF53A; actin-like 6A	2.006
SUV39H1	suppressor of variegation 3-9 homolog 1 (Drosophila)	2.006
BATF	basic leucine zipper transcription factor, ATF-like	2.004
MIB2	mindbomb E3 ubiquitin protein ligase 2	2.003
GPR56	G protein-coupled receptor 56	2.000
TPPP3	tubulin polymerization-promoting protein family member 3	-2.000
SWAP70	SWAP switching B-cell complex 70kDa subunit	-2.001
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	-2.003
RNF130	GOLIATH; ring finger protein 130	-2.013
CD300LB	CLM7; TREM5; IREM3	-2.015
TLR10	toll-like receptor 10	-2.016
LAMC1	laminin, gamma 1 (formerly LAMB2)	-2.020
FCAR	CD89; Fc fragment of IgA, receptor for	-2.021
VCAN	versican	-2.021
LRP3	low density lipoprotein receptor-related protein 3	-2.026
AFF3	LAF4; AF4/FMR2 family, member 3	-2.029
TNFRSF4	OX40; CD134	-2.034
ZNF395	PBF; HDBP2	-2.036
CFD	DF; FD; complement factor D (adipsin)	-2.042
GNG7	guanine nucleotide binding protein (G protein), gamma 7	-2.043
RAB31	Rab22B; RAB31, member RAS oncogene family	-2.043
MEF2C	myocyte enhancer factor 2C	-2.044
VWF	von Willebrand factor	-2.048
CBX7	chromobox homolog 7	-2.056
GALNAC4S-6ST	CHST15; BRAG; carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	-2.057
S100A12	ENRAGE; S100 calcium binding protein A12	-2.059
TSPAN9	tetraspanin 9	-2.061
TCEA3	TFIIS; transcription elongation factor A (SII), 3	-2.065
RASD1	AGS1; DEXRAS1; RAS, dexamethasone-induced 1	-2.068
MPL	TPOR; myeloproliferative leukemia virus oncogene	-2.074
PROK2	BV8; prokineticin 2	-2.077
GPR162	G protein-coupled receptor 162	-2.079
CCR7	chemokine (C-C motif) receptor 7	-2.080
SSBP2	single-stranded DNA binding protein 2	-2.081
TRIB1	tribbles homolog 1 (Drosophila)	-2.084
ZFHX3	ATBF1; zinc finger homeobox 3	-2.089
CD79B	IGB; CD79b molecule, immunoglobulin-associated beta	-2.093
BLK	B lymphoid tyrosine kinase	-2.095
SLC26A11	solute carrier family 26, member 11	-2.095
DENND2D	DENN/MADD domain containing 2D	-2.096
PTCRA	pre T-cell antigen receptor alpha	-2.098
ANG	angiogenin, ribonuclease, RNase A family, 5	-2.106
CXCL16	SRPSOX; chemokine (C-X-C motif) ligand 16	-2.110

SPINT2	HAI-2; serine peptidase inhibitor, Kunitz type, 2	-2.112
DAB2	disabled homolog 2	-2.121
NUAK2	SNARK; NUAK family, SNF1-like kinase, 2	-2.123
ITGB5	integrin, beta 5	-2.124
TCEAL3	transcription elongation factor A (SII)-like 3	-2.127
MOSC1	MARC1; mitochondrial amidoxime reducing component 1	-2.130
RHOB	ras homolog family member B	-2.132
LOC90925	IGHV5-78; immunoglobulin heavy variable 5-78 (pseudogene)	-2.134
LRRC26	CAPC; leucine rich repeat containing 26	-2.136
MGC13057	chromosome 2 open reading frame 88	-2.136
SOCS2	suppressor of cytokine signaling 2	-2.136
MERTK	c-mer proto-oncogene tyrosine kinase	-2.137
ACTN1	actinin, alpha 1	-2.138
DDIT4	Dig2; REDD1; DNA-damage-inducible transcript 4	-2.146
ZNF467	EZI; zinc finger protein 467	-2.156
D4S234E	NSG1; NEEP21; neuron specific gene family member 1	-2.157
MGST1	microsomal glutathione S-transferase 1	-2.160
VNN3	vanin 3	-2.161
EGR2	early growth response 2	-2.172
FLJ43093	RAB44; RASD3; RASL13; member RAS oncogene family	-2.172
DPEP2	dipeptidase 2	-2.183
RTN1	reticulon 1	-2.190
SVIL	supervillin	-2.194
DEF8	differentially expressed in FDCP 8 homolog (mouse)	-2.196
GPR177	EVI; MRP; GPR177	-2.196
FCRL2	Fc receptor-like 2	-2.198
NELL2	NEL-like 2 (chicken)	-2.201
TBC1D9	MDR1; TBC1 domain family, member 9 (with GRAM domain)	-2.227
FAM134B	JK1; family with sequence similarity 134, member B	-2.233
CD163	CD163 molecule	-2.236
IL11RA	interleukin 11 receptor, alpha	-2.236
CD93	C1qRP;CD93 molecule	-2.247
SPRED1	sprouty-related, EVH1 domain containing 1	-2.248
RGS2	regulator of G-protein signaling 2, 24kDa	-2.250
PDE5A	CN5A; PDE5; phosphodiesterase 5A, cGMP-specific	-2.251
TGFBI	transforming growth factor, beta-induced, 68kDa	-2.253
CLEC4A	DCIR; C-type lectin domain family 4, member A	-2.259
C7orf41	chromosome 7 open reading frame 41	-2.268
MS4A6A	membrane-spanning 4-domains, subfamily A, member 6A	-2.273
TPM2	tropomyosin 2 (beta)	-2.273
RBP7	CRBPIV; retinol binding protein 7, cellular	-2.283

PTGDS	PGDS; LPGDS; prostaglandin D2 synthase 21kDa (brain)	-2.288
CD19	CD19 molecule	-2.290
CXCR4	chemokine (C-X-C motif) receptor 4	-2.292
RXRA	retinoid X receptor, alpha	-2.297
BASP1	brain abundant, membrane attached signal protein 1	-2.308
CACNA1I	Cav3.3; calcium channel, voltage-dependent, T type, alpha 1I subunit	-2.315
COBLL1	COBL-like 1	-2.335
NLRP12	NALP12; NLR family, pyrin domain containing 12	-2.336
TSPAN18	tetraspanin 18	-2.343
NR4A3	nuclear receptor subfamily 4, group A, member 3	-2.344
KBTBD11	kelch repeat and BTB (POZ) domain containing 11	-2.349
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	-2.350
LTB	lymphotoxin beta (TNF superfamily, member 3)	-2.366
CSF3R	CD114; GCSFR; colony stimulating factor 3 receptor (granulocyte)	-2.367
MAL	mal, T-cell differentiation protein	-2.371
FCGBP	Fc fragment of IgG binding protein	-2.376
RNASE4	ribonuclease, RNase A family, 4	-2.386
AKR1C3	aldo-keto reductase family 1, member C3	-2.396
LRRN3	NLRR3; leucine rich repeat neuronal 3	-2.397
VENTX	VENT homeobox	-2.397
CXCR5	chemokine (C-X-C motif) receptor 5	-2.405
CNTNAP2	contactin associated protein-like 2	-2.417
ZSCAN18	zinc finger and SCAN domain containing 18	-2.449
BANK1	B-cell scaffold protein with ankyrin repeats 1	-2.451
C5orf29	GAPT; GRB2-binding adaptor protein, transmembrane	-2.496
TCL1A	TCL1; T-cell leukemia/lymphoma 1A	-2.503
CYBRD1	cytochrome b reductase 1	-2.508
ZDHHC1	zinc finger, DHHC-type containing 1	-2.512
BCL2	B-cell CLL/lymphoma 2	-2.513
LY86	MD-1; lymphocyte antigen 86	-2.523
PDK4	pyruvate dehydrogenase kinase, isozyme 4	-2.527
OSBPL10	ORP10; OSBP9; oxysterol binding protein-like 10	-2.529
AXIN2	axin 2	-2.543
VIPR1	VPAC1; vasoactive intestinal peptide receptor 1	-2.550
CHST13	C4ST3; carbohydrate (chondroitin 4) sulfotransferase 13	-2.553
CD83	CD83 molecule	-2.570
RGS18	regulator of G-protein signaling 18	-2.572
SLC40A1	FPN1; solute carrier family 40 (iron-regulated transporter), member 1	-2.601
VPREB3	pre-B lymphocyte 3	-2.606
F13A1	coagulation factor XIII, A1 polypeptide	-2.651
CD79A	MB-1; IGA; CD79a molecule, immunoglobulin-associated alpha	-2.713

FCRLA	FREB; Fc receptor-like A	-2.716
PTGS1	COX1; prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	-2.721
PPBP	CXCL7; Beta-TG; NAP-2	-2.747
SH3BGRL2	SH3 domain binding glutamic acid-rich protein like 2	-2.754
PADI4	PAD4; peptidyl arginine deiminase, type IV	-2.836
EBI2	GPR183; G protein-coupled receptor 183	-2.850
C5AR1	C5A; C5AR; CD88; complement component 5a receptor 1	-2.873
THBS1	TSP1; thrombospondin 1	-2.882
RNASE6	ribonuclease, RNase A family, k6	-2.896
GP9	GPIX; glycoprotein IX (platelet)	-2.923
NR4A2	NURR1; nuclear receptor subfamily 4, group A, member 2	-2.954
CD1C	BDCA1; CD1c molecule	-2.959
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	-3.000
CD24	CD24 molecule	-3.025
CXCL2	MIP-2	-3.045
G0S2	G0/G1switch 2	-3.059
SGK	serum/glucocorticoid regulated kinase 1	-3.260
SDPR	serum deprivation response	-3.273
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	-3.480
IRS2	insulin receptor substrate 2	-3.557
HBEGF	heparin-binding EGF-like growth factor	-3.833
OSM	oncostatin M	-4.324
IER3	IEX1 or immediate early response gene 3	-4.398
IL1B	Interleukin – 1beta	-4.520
IL8	Interleukin - 8	-5.197
PTGS2	COX2	-5.360

Table A-1. Shows the complete list of 464 genes significantly up/down regulated during acute infection with EBV, the common and/or official name of the gene, as well as the average fold change within the EBV cohort. The genes shown were derived from analysis of EBV subjects (fold change ≥ 2 and met a statistical significance cutoff of p-value of ≤0.05 with Bonferonni multiple-tests correction).

Group	Gene Symbol	Common and/or Official Name
EBV unique	CHI3L2	YKL-39 or chitinase 3-like 2
	CD8A	CD8
	GZMH	granzyme H
In common with DENV	CD38	CD38
	LMNB1	lamin B1
	APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial
	LAG3	lymphocyte-activation gene 3
	MT1E	metallothionein 1E
	E2F2	E2F transcription factor 2
	CKS2	CDC28 protein kinase regulatory subunit 2
	ASF1B	ASF1 anti-silencing function 1 homolog B or CIA-II
	ACOT7	BACH or acyl-CoA thioesterase 7
	MCM6	minichromosome maintenance complex component 6
	C18orf56	chromosome 18 open reading frame 56
	CCNB2	cyclin B2
	CDT1	chromatin licensing and DNA replication factor 1
	EXO1	exonuclease 1
	AURKB	aurora kinase B
	BUB1	budding uninhibited by benzimidazoles 1
	CCNF	cyclin F or FBX1
	KIFC1	kinesin family member C1
	TIMD4	TIM4 or T-cell immunoglobulin and mucin domain containing 4
	CDCA7	JPO1 or cell division cycle associated 7
	MND1	meiotic nuclear divisions 1 homolog
	SLC27A2	solute carrier family 27 (fatty acid transporter), member 2
	GINS2	GINS complex subunit 2
	KIAA0101	PAF, PAF15, p15PAF, or p15(PAF)
	TYMS	TS or thymidylate synthetase
	STMN1	stathmin 1
	PCNA	proliferating cell nuclear antigen
	GMNN	geminin, DNA replication inhibitor
	ATAD2	ANCCA or ATPase family, AAA domain

		containing 2
	CENPN	centromere protein N
	CDC20	cell division cycle 20 homolog
	CDCA3	cell division cycle associated 3
	TPX2	microtubule-associated, homolog
	AURKA	aurora A or aurora kinase A
	KIF14	kinesin family member 14
	HJURP	Holliday junction recognition protein
	FANCI	Fanconi anemia, complementation group I
	CDCA8	BOREALIN or cell division cycle associated 8
	BIRC5	baculoviral IAP repeat containing 5
	CKAP2L	cytoskeleton associated protein 2-like
	CDCA2	Repo-Man or cell division cycle associated 2
	KIF2C	MCAK
	POLQ	polymerase (DNA directed), theta
	CDC45L	cell division cycle 45 homolog
	CDCA5	SORORIN or cell division cycle associated 5
	UBE2C	ubiquitin-conjugating enzyme E2C
	MCM2	minichromosome maintenance complex component 2
	TK1	thymidine kinase 1, soluble
	CCNA2	cyclin A2
	ASPM	asp (abnormal spindle) homolog, microcephaly associated
	APOBEC3H	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3H
	SPC24	NDC80 kinetochore complex component, homolog
	CDC2	CDK1 or cyclin-dependent kinase 1
	HMMR	RHAMM or hyaluronan-mediated motility receptor
	MCM10	minichromosome maintenance complex component 10
	OIP5	LINT-25
	C12orf48	PARPBP or PARI
	WDR51A	POC1A or PIX2
	MCM4	minichromosome maintenance complex component 4
	PLK4	polo-like kinase 4 or SAK
	NUSAP1	nucleolar and spindle associated protein 1
	TOP2A	topoisomerase (DNA) II alpha
	CENPA	centromere protein A
	CHEK1	checkpoint kinase 1 or CHK1
	STIL	SCL/TAL1 interrupting locus or SIL
	CENPM	centromere protein M

	CDKN3	cyclin-dependent kinase inhibitor 3 or KAP1
	NCAPG	non-SMC condensin I complex, subunit G
	PTTG3	PTTG3P or pituitary tumor-transforming 3, pseudogene
	TTK	MPS1
	FEN1	flap structure-specific endonuclease 1
	PTTG1	pituitary tumor-transforming 1
	MELK	maternal embryonic leucine zipper kinase
	DLG7	HURP or DLGAP5
	KIF11	EG5
	KIF15	HKLP2
	CEP55	centrosomal protein 55kDa
	KIF20A	MKLP2
	UBE2T	ubiquitin-conjugating enzyme E2T
	POLE2	polymerase (DNA directed), epsilon 2, accessory subunit
	TRIP13	thyroid hormone receptor interactor 13
	PSAT1	phosphoserine aminotransferase 1
	PHGDH	phosphoglycerate dehydrogenase
	PRC1	protein regulator of cytokinesis 1
	RAD51AP1	RAD51 associated protein 1
	RFC4	replication factor C (activator 1) 4
	IFNG	interferon, gamma
	TUBA1C	tubulin, alpha 1c
Common	C1QB	complement component 1, q subcomponent, B chain
	IFI27	interferon, alpha-inducible protein 27
	ANKRD22	ankyrin repeat domain 22
	CXCL10	IP-10
	GBP4	guanylate binding protein 4
	GBP5	guanylate binding protein 5
	GBP1	guanylate binding protein 1, interferon-inducible
	STAT1	signal transducer and activator of transcription 1
	MT2A	metallothionein 2A
	OASL	2'-5'-oligoadenylate synthetase-like
	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9
Down in Some	IER3	IEX1 or immediate early response gene 3
	G0S2	G0/G1switch 2
	CXCL2	MIP-2
	IL1B	interleukin – 1beta
	IL8	interleukin - 8
	PTGS2	COX2

	HBEGF	heparin-binding EGF-like growth factor
	OSM	oncostatin M
	IRS2	insulin receptor substrate 2
	SGK	serum/glucocorticoid regulated kinase 1
	SDPR	serum deprivation response

Table A-2. Genes with a fold change ≥ 3.

Gene Symbol	Common and/or Official Name
ACTN1	actinin, alpha 1
ALS2CR4	TMEM237
ANG	angiogenin, ribonuclease, RNase A family, 5
BATF	basic leucine zipper transcription factor, ATF-like
C16ORF59	chromosome 16 open reading frame 59
C17ORF53	chromosome 17 open reading frame 53
C18ORF24	SKA1; spindle and kinetochore associated complex subunit 1
C18ORF56	chromosome 18 open reading frame 56
C1ORF112	chromosome 1 open reading frame 112
C1ORF135	AUNIP
C9ORF140	SAPCD2
CCNF	cyclin F; FBX1
CD300LB	CD300 molecule-like family member b
CHAF1B	CAF1; chromatin assembly factor 1, subunit B (p60)
CNTNAP2	contactin associated protein-like 2
CXCL9	chemokine (C-X-C motif) ligand 9
DCLRE1A	SNM1; SNM1A; DNA cross-link repair 1A
DDX39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A
DEF8	differentially expressed in FDCP 8 homolog
DHRS3	dehydrogenase/reductase (SDR family) member 3
DSN1	MIND kinetochore complex component, homolog
ENDOGL1	ENDOGL1; endo/exonuclease (5'-3'), endonuclease G-like
FLJ33590	CXXC11
GINS4	GINS complex subunit 4
GP9	GPIX; glycoprotein IX
GPR162	G protein-coupled receptor 162
HMGN2	high mobility group nucleosomal binding domain 2
IL32	interleukin 32
INCENP	inner centromere protein antigens 135/155kDa
KCNK10	TREK2; potassium channel, subfamily K, member 10
KIF19	kinesin family member 19
LOC90925	IGHV5-78
LRP3	low density lipoprotein receptor-related protein 3
MCOLN2	TRPML2
MRPL10	mitochondrial ribosomal protein L10
NLRP12	NALP12; NLR family, pyrin domain containing 12
OBFC2B	NABP2; SSB1
PAQR4	progestin and adipoQ receptor family member IV
PDCD1	PD1; programmed cell death 1

PGAM1	phosphoglycerate mutase 1
PIF1	PIF1 5'-to-3' DNA helicase homolog
PPP1CA	protein phosphatase 1, catalytic subunit, alpha isozyme
PRR11	proline rich 11
PTCRA	pre T-cell antigen receptor alpha
RALY	RNA binding protein, autoantigenic (hnRNP-associated with lethal yellow homolog
SPINT2	HAI-2
SPRED1	sprouty-related, EVH1 domain containing 1
SSBP2	SSBP2 single-stranded DNA binding protein 2
SVIL	supervillin
TCEAL3	transcription elongation factor A (SII)-like 3
TLR10	toll-like receptor 10
TNFRSF4	OX40
TSPAN18	tetraspanin 18
TSPAN9	tetraspanin 9
TXNL2	PICOT; GRX3; glutaredoxin 3
USP54	ubiquitin specific peptidase 54
VENTX	VENT homeobox
VWF	von Willebrand factor
ZDHHC1	zinc finger, DHHC-type containing 1

Table A-3. Lists genes changed during EBV infection with a fold change ≥ 2 and a *p* value of ≤ 0.05, but also had an average fold change < 1.5 in all other viral infections. The gene symbol and the common or official name for the gene are shown.