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Nicole Brock

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HOST-SPECIFIC PLASMACYTOID DENDRITIC CELL DEFENSES IN THE PRESENCE OF HUMAN AND MACAQUE SKIN CELLS INFECTED WITH B VIRUS

by

NICOLE BROCK

Under the Direction of Julia K. Hilliard

ABSTRACT

Plasmacytoid dendritic cells (pDC) are a specialized group of circulating dendritic cells that respond to viral nucleic acids with Type I IFN production as well as other cytokine and chemokines. These pDC responses lead to the production of antiviral molecules and recruitment of defense cells. During zoonotic B virus infection, a simplex virus of the subfamily Alphaherpesviridae, our lab has observed that infected individuals who succumb to infection have little-to-no-antibody or cell-mediated defenses. To identify whether this was partly due to failure of pDCs to produce antiviral interferon responses or produce chemokine and cytokines, we tested the hypothesis that B virus modulates the IFN response during zoonotic infection by blocking pDC activation and subsequent IFN signaling pathways to circumvent host defenses, while these pathways remain intact in the macaque hosts. We showed that human pDCs respond to B virus through the production of IFN-α, IL-1α, IL-6, TNF-α, MIP-1α/β and IP-10. Human pDCs co-cultured with B virus infected fibroblasts produced fewer cytokines and at lower levels. The macaque response to B virus was measured using PBMCs, as there are no specific reagents available
to enrich macaque pDCs. Human and macaque PBMCs produced IFN-α when exposed directly to B virus infected lysates. Co-cultures of PBMCs with B virus infected fibroblasts from both hosts failed to produce any significant amounts of IFN-α. To quantify the antiviral effects of PBMC induced IFN-α, we measured B virus titers after exposure to supernatants from B virus exposed PBMCs, PBMC co-cultures with infected fibroblasts and exogenous recombinant Type I IFN. Our data further suggest that B virus resistance was not due to virus specific blockade of the Type I IFN signaling pathway because STAT-1 was activated in infected fibroblasts when treated with Type I IFNs. These data demonstrate for the first time that B virus replication is unimpeded in the presence of any source of IFN-α in either host cell type. In conclusion, this dissertation shows that the IFN-α production by both hosts in response to B virus is similar and that IFN-α treatment of B virus infected fibroblasts did not reduce B virus replication.

INDEX WORDS: Plasmacytoid dendritic cells, Interferon, Fibroblasts, Macaque, Herpes B virus, *Macacine herpesvirus* 1, STAT-1, Innate immunity
HOST-SPECIFIC PLASMACYTOID DENDRITIC CELL DEFENSES IN THE PRESENCE OF HUMAN AND MA-
CAQUE SKIN CELLS INFECTED WITH B VIRUS

by

NICOLE BROCK

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2014
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by

NICOLE BROCK

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Committee: Richard Dix
Yuan Liu

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
May 2014
DEDICATION

This dissertation is dedicated to the loves of my life; to Josh, without your unfailing support, love and wisdom I would not have completed this journey. Thank you for always being there for me and for your advice that was sometimes difficult to hear. Thank you for pushing me to excel no matter the odds or circumstances. Also, thank you for making sacrifices so that I could pursue my dream.

This dissertation is also dedicated to my children; Shelby, you were created at the beginning of this journey and have given me the desire to be a strong role model and someone you can look up to; to Lilia for teaching me patience in overcoming tough challenges and showing me how to fall down and get back up; and to Aengus, for teaching me to appreciate and embrace the unexpected.

I love you all beyond the edges of the universe. Thank you for being my motivation, my drive, my heart, and my soul.

“When we love, we always strive to become better than we are. When we strive to become better than we are, everything around us becomes better too.” Paulo Coelho
ACKNOWLEDGEMENTS

First, I’d like to acknowledge my mentor Dr. Julia Hilliard. Thank you for your mentorship, life advice, patience and flexibility. I appreciate all that you have done for me.

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LIST OF ABBREVIATIONS

Ag: antigen
AIM2: absent in melanoma 2
ANOVA: analysis of variance
APC: antigen presenting cell
ASC: apoptosis associated Speck like protein containing a carboxy terminal CARD
ATP: adenosine triphosphate
BDCA: blood dendritic cell antigen
BV: B virus, Herpes B virus
c-GAMP: cyclic guanosine monophosphate adenosine monophosphate
c-GAS: c-GAMP synthase
CARD: caspase activation and recruitment domain
CD: cluster of differentiation
CDC: Center for Disease Control
CNS: central nervous system
CpG/ CpG ODN: unmethylated C+G DNA oligonucleotides
DAI/ZBP1: DNA dependent activator of IFN regulatory factors
DAMP: damage associated molecular pattern
DC: dendritic cell
DDX: DexD/H box
DMEM: Dulbecco’s minimal essential media
DMSO: dimethyl sulfoxide
DNA-PK: DNA dependent protein kinase C
DNA: deoxyribonucleic acid
DRG: dorsal root ganglia
ECL: enhanced chemiluminescence
ECM: extracellular matrix
EDTA: Ethylene diaminetetra acetic acid
ELISA: enzyme-linked immunosorbent assay
FBS: fetal bovine serum
G-CSF: granulocyte colony stimulating factor
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GM-CSF: granulocyte macrophage colony stimulating factor
gX: glycoprotein X
HFF: human foreskin fibroblasts
HIV: human immunodeficiency virus
HMBG1: non-histone chromatin associating protein high mobility group box 1
HSP: heat shock protein
HSV: herpes simplex virus
HVEM: herpesvirus entry mediator
IC_{50}: half maximal inhibitory concentration
ICOS: Inducible T-cell co-stimulator
ICP0: infected cell protein 0
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFI16</td>
<td>IFNγ inducible protein 16</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNαR</td>
<td>Type I interferon receptor</td>
</tr>
<tr>
<td>Igs</td>
<td>immunoglobulins</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of kappa light polypeptide gene enhancer in B cells, kinases</td>
</tr>
<tr>
<td>IL-1R</td>
<td>interleukin 1 receptor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILT</td>
<td>immunoglobulin like transcript</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon γ induced protein 10</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>ISGF</td>
<td>interferon stimulated gene factor</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>LGP2</td>
<td>laboratory of genetics and physiology 2</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MCL</td>
<td>mock cell lysate</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation associated protein 5</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NETS</td>
<td>neutrophil extracellular traps</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide oligomerization and binding domain (NOD) like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide oligomerization and binding domain</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PILRα</td>
<td>paired immunoglobulin-like type 2 receptor alpha</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PRR</td>
<td>pathogen recognition receptor</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RIG I</td>
<td>retinoic acid-inducible gene 1</td>
</tr>
<tr>
<td>RLR</td>
<td>retinoic inducible gene I (RIG-I) like receptor</td>
</tr>
<tr>
<td>RMF</td>
<td>rhesus macaque fibroblasts</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error mean</td>
</tr>
</tbody>
</table>
STAT: signal transduction activator of transcription
STING: stimulator of IFN genes
SUP: supernatant
T reg: T regulatory cell
TANK: TRAF family member associated NFκB activator
TBK: tank binding kinase
TH: T helper
TICAM1: toll-like receptor adaptor molecule 1
TIR: toll/interleukin 1 receptor
TIRAP: TIR associated protein
TK: thymidine kinase
TLR: toll-like receptor
TNFR: tumor necrosis factor receptor
TRAF6: TNFR associated factor 6
TRAM: TRIF related adaptor molecule
TRIF: TIR domain containing adaptor protein inducing IFN β
INTRODUCTION

1.1 Literature Review

1.1.1 B virus

B virus, or *Macacine herpesvirus* 1, formerly known as *Cercopithicene herpesvirus* 1, was first discovered in 1934 when a doctor named Dr. William B. Brebner, was exposed to contaminated tissue during necropsy of a seemingly healthy macaque. Dr. Brebner showed relatively mild symptoms early in infection until day 10, where symptoms of central nervous system involvement were apparent. His symptoms became increasingly worse and he succumbed to infection 15 days after the onset of neurological involvement. Post-mortem analysis indicated death due to acute disseminated disease and encephalomyelitis. Frederick Gay and Margaret Holden were one of two groups to first to identify the agent as viral by injecting brain and spinal cord suspensions from Dr. Brebner into rabbits. The disease in rabbits mimicked that of the disease in Dr. Brebner, although the rabbits succumbed in four to five days post-infection. They designated the agent W virus [1, 2]. Colleagues within their same department, Drs. Sabin and Wright, also identified the agent as a herpes virus around the same time, described the same disease in rabbits and designated it B virus [3].

B virus has a linear double-stranded DNA genome of 157 kpb [4], although previous studies prior to sequencing reported the length at 162.5 kbp [5] and 165 kbp [6] with 74.5% G+C composition [4]. The genome is surrounded by an icosahedral capsid with T=16 symmetry and a diameter of about 40nm [7], which is surrounded by tegument proteins derived from host cell protein as well as virally encoded proteins. The tegument is surrounded by a double-membrane obtained from the viral glycoprotein studded host plasma membrane following exocytosis from the cell. The complete virus particle is approximately 160-180 nm in diameter [8].
The B virus genome is predicted to encode up to 74 proteins [4]. At least 11 of these have been identified as viral glycoproteins [9]. Genome sequence analysis predicted the presence of these eleven glycoproteins as homologs of HSV-1 and HSV-2: gL [4, 10, 11], gM [4, 11], gB [4, 11], gC [4, 11], gH [4, 11], gG [4, 10, 12], gJ [4, 10, 13], gD [4, 10, 13], gL [4, 10], gE [4, 10], and gK [4, 11]. Recombinant proteins have been produced for gB, gC, gD gE and the secreted and membrane-associated segments of gG [14].

B virus enters target cells primarily by fusion as well as receptor-mediated endocytosis, similar to HSV-1/2 (Figure 1) [15] and I. Patrusheva, et al, unpublished raw data. B virus uses the common entry receptors as HSV-1/2, heparan sulfate and nectin-1, but does not use the paired immunoglobulin like receptor-α (PILR-α) or herpesvirus entry mediator (HVEM) [16]. It has also been shown that B virus can use other receptors in addition to these, which may shed some light on the pathogenesis of B virus in humans (I. Patrusheva, et al, unpublished raw data). Recent data has shown that gD is not required for B virus entry into certain cells types, which is a unique difference between B virus and the human simplex viruses to which it is closely related (I. Patrusheva, et al, unpublished raw data). Figure 1 is a working model demonstrating the known mechanisms of entry for HSV, some of which are shared by B virus.

B virus, like all herpes viruses, replicates and assembles in the nucleus of infected cells. After virus entry into cells, the capsid and the tegument are transported to the nuclear pores where the DNA genome is released into the nucleus. Viral gene transcription takes place within the nucleus and is mediated by host RNA polymerase II (pol II) along with viral and host transcription factors. As previously stated, B virus is predicted to encode about 74 genes (Table 1) [4]. B virus transcription occurs in a cascade fashion beginning with the production of immediate early genes (α), which mediate the transcription of early genes (β) which then allow the transcription of the late genes (γ) and DNA replication. The immediate early genes are transcribed without the production of newly formed virus proteins, so
Figure 1 Herpes simplex virus entry pathways

HSV can enter cells a variety of ways depending on a number of cellular and viral factors [15].

must rely on host cell proteins and viral tegument proteins. A few examples of the immediate early genes include infected cell protein 0 (ICP0) which has numerous functions, but a few include modulation of host innate immune responses and promoting virus replication. The early genes products peak around 5-7 h post-infection and include enzymes and DNA-binding proteins involved in replication including thymidine kinase (TK). Late gene transcription is dependent on virus replication and these gene products are primarily involved in virion assembly and include the glycoproteins and capsid proteins (Figure 2). The late genes can be divided into two sets of γ1 and γ2, depending on the timing of their transcription. The γ1 genes are referred to as leaky late genes, as expression in minimally affected by inhibition of replication, while the γ2 genes, or true late genes, are exclusively dependent on DNA replication as inhibition prevents their transcription [17]. There are only a small number of differences discovered thus far between B virus and HSV-1 and HSV-2 [4]. Two of these differences are noteworthy and were discovered in our laboratory. One is the absence of a protein that has been implicated to have
multiple functions, most predominantly neurovirulence, i.e., γ34.5 [18, 19]. A second noted difference was noted due to the absence γ34.5, which also plays a role in blocking the innate defenses involved with the activation of protein kinase RNA-activated (PKR), activated by Type 1 interferons. B virus has selected for an alternative viral protein, Us11, to block this pathway, a function that can occur as a result of early expression of B virus Us11 [20]. HSV Us11 proteins are not expressed until late in infection [21], thus it appears that the novel temporal expression of B virus Us11 compensates for the lack of γ34.5 homologs.

**Figure 2** Herpes virus replication cycle
After entry into the cell and DNA delivery to the nucleus, the virus begins transcription of viral genes in a cascade of α, β, and γ genes [22].
The natural hosts for B virus are members of the *Macaca* species of Old World monkeys. The disease within the natural host is relatively mild and similar to HSV-1/2 disease in humans, with occasional oral or genital lesions, although acute infection, as with HSV-1/2 can also occur with no apparent lesions. B virus replicates in the mucosal epithelial cells, causing focal necrosis (lesion), where it gains access to the peripheral nerves innervating the site of injury/entry. The virus travels within a neuron via retrograde transport mechanism, and then establishes latency in the dorsal root or cranial ganglia. B virus typically reactivates in macaques during mating season, monsoon seasons and during times of stress, such as transport from facilities or the wild [23, 24] (Farah-Abraham et al., unpublished raw data).
Table 1 Predicted Open Reading Frames of B virus

Genetic sequence analysis of B virus E2490 has predicted a number of genes and their functions by comparison with HSV-1 and 2 [4].

<table>
<thead>
<tr>
<th>ORF or feature</th>
<th>Location</th>
<th>Strand</th>
<th>Length (codons)</th>
<th>Identity (%)</th>
<th>Characteristics and predicted function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR1</td>
<td>1</td>
<td>+</td>
<td>223</td>
<td>Terminal direct repeat</td>
<td>Terminal copy of large repeat region</td>
</tr>
<tr>
<td>RL2</td>
<td>1</td>
<td>+</td>
<td>9021</td>
<td>Immediate-early protein (ICP0); multifunctional regulatory protein</td>
<td></td>
</tr>
<tr>
<td>Exon 1</td>
<td>2194</td>
<td>+</td>
<td>2241</td>
<td>43.5%</td>
<td>40.1%</td>
</tr>
<tr>
<td>Exon 2</td>
<td>2461</td>
<td>+</td>
<td>3003</td>
<td>61.8%</td>
<td>61.8%</td>
</tr>
<tr>
<td>Exon 3</td>
<td>3125</td>
<td>+</td>
<td>4636</td>
<td>56.1%</td>
<td>56.5%</td>
</tr>
<tr>
<td>UL1</td>
<td>9022</td>
<td>+</td>
<td>11683</td>
<td>Unique large region</td>
<td></td>
</tr>
<tr>
<td>UL2</td>
<td>9072</td>
<td>+</td>
<td>9746</td>
<td>325</td>
<td>65.5% 53.4</td>
</tr>
<tr>
<td>UL3</td>
<td>1071</td>
<td>+</td>
<td>11394</td>
<td>Capid protein; DNA cleavage/packaging</td>
<td></td>
</tr>
<tr>
<td>UL4</td>
<td>12120</td>
<td>+</td>
<td>11509</td>
<td>Capid protein; DNA cleavage/packaging</td>
<td></td>
</tr>
<tr>
<td>UL5</td>
<td>14820</td>
<td>+</td>
<td>12172</td>
<td>Component of helicase-primase complex</td>
<td></td>
</tr>
<tr>
<td>UL6</td>
<td>14819</td>
<td>+</td>
<td>16870</td>
<td>Component of helicase-primase complex</td>
<td></td>
</tr>
<tr>
<td>UL7</td>
<td>10821</td>
<td>+</td>
<td>17711</td>
<td>O/A binding protein; helicase activity</td>
<td></td>
</tr>
<tr>
<td>UL8</td>
<td>20717</td>
<td>+</td>
<td>17886</td>
<td>76.2% 60.4%</td>
<td></td>
</tr>
<tr>
<td>UL9</td>
<td>22447</td>
<td>+</td>
<td>20223</td>
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Table 1 (cont.) Predicted Open Reading Frames of B virus

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Upon reactivation, B virus travels via anterograde transport; and upon re-entry into the mucosal epithelium begins the replication cycle again and the production of infectious virions for infection of new hosts or to freshen the supply of virus establishing latency in the dorsal root or cranial ganglia (Figure 3). Fatal B virus infection in macaques is rare and is usually seen when a monkey is immunosuppressed [24, 25]. Other species of monkeys are also susceptible to fatal B virus infection, when they come into contact with B virus shedding macaques, observed and reported in patas monkeys [26], colobus monkeys [26], capuchins [2], and common marmosets [27]. Infection usually occurs when these monkeys are co-housed with infected macaques. B virus-infected cell cultures show the characteristic feature of herpes viruses in that they form syncytia or multi-nucleated cells [28], particularly in epithelial cells derived
from non-macaque species (Figures 4 and 5). This allows for the virus to maximize productivity and limit the amount of cell-free virions.

In contrast, human infection (zoonotic) is fatal in up to 80% of cases if not treated quickly enough following infection. B virus is the only herpesvirus thought thus far to be capable of zoonotic infection [29]. Humans are typically infected from a bite or scratch from an infected monkey, or from mucosal exposure to infected tissues or secretions. B virus replicates at the site of entry, in the periphery, causing edema and swelling and the characteristic herpetic lesions in some cases. B virus enters the peripheral nerves innervating the site of injury or exposure, and travels to the sensory root ganglia. The unusual and striking characteristic with human disease is that the virus travels beyond the DRG or cranial ganglia, and enters the central nervous system (CNS), where it travels to the brainstem via the dorsal horn of the spinal cord or from the cranial ganglia, usually the ophthalmic branch of the trigeminal nerve in the case of eye splash exposures. Whether virus-directed destruction or immune defenses mediate the neurological symptoms seen with most fatal human infections remains unknown. Ascending paralysis results in respiratory failure. After B virus has entered the CNS, it is often impossible to curtail the progression of disease. Reactivation from surviving human hosts has been observed in a few cases and there has been one case of observed human-to-human transmission [30].
Figure 3 Life cycle of herpes viruses in their natural hosts
Herpes viruses replicate in the periphery and gain access to the peripheral neurons innervating the area. The virions are transported to ganglia where the virus can then establish latency. During reactivation, virus travels to the periphery to initiate lytic infection [31]. In human B virus infection, virus continues to the CNS, leading to encephalitis, encephomyelitis and death.

Figure 4 Morphology of B virus infection in monkey epithelial cells
An electron microscopic image of monkey epithelial cell (Vero) infected with B virus at 24h post-infection. There are numerous budding particles on the cell surface (→) and the chromatin has dissipated (*) [28].
Figure 5 B virus infection in monkey epithelial cells at different times post-infection

The characteristic syncytia caused by B virus infection in monkey cells at different times post-infection. (1) Normal uninfected cells, (2) at 6 h post-infection, the nucleolus begins to dissolve (→), and (3, 4) at 36 h post-infection, multinucleated cells (→) are observed [28].

Diagnosis of B virus infection in humans and macaques is accomplished by detection of serum antibodies reactive to B virus in Western blot assays and ELISA because virus is rarely shed peripherally. Polymerase Chain Reaction (PCR) is used for the detection of B virus DNA, and cell-culture methods for the detection of infectious particles, but these methods produce successful only when virus is being shed into a sampled site, e.g., tears, saliva, genital secretions, etc. B virus can also be present in fomite contaminants. Diagnosis in macaques can be difficult, because while the virus is latent, animals may not have detectable levels of serum antibodies against B virus, particularly during primary infections. A difficulty in diagnosing humans is the fact that B virus antibodies cross react with HSV-1/2 antibodies, making a positive B virus diagnosis for co-infected individuals challenging [32-34].
While there is no cure for B virus, rapid treatment following exposure can increase the survival rate to nearly 80%. Of course, thorough and immediate cleansing is the key to the prevention of B virus infection. Prophylaxis may have an added benefit of reducing the amount of virus that reaches the dorsal root or cranial ganglia [32]. After a fatal case of zoonotic infection resulting from an ocular splash in 1997, the Center for Disease Control (CDC) recommended the use of goggles and masks for individuals working where macaques are or have been recently. The first few minutes after a potential B virus exposure are crucial to preventing disease. The person should wash the wound or site of exposure thoroughly for at least 15 min. Post-exposure prophylaxis is nearly always recommended (Table 2) [32]. Currently, there are three available drugs for the treatment of B virus including acyclovir, valacyclovir, and famciclovir, with acyclovir or valacyclovir preferred for patients who have not manifested CNS symptoms. Gancyclovir is recommended for zoonotic infections that have progressed to CNS symptoms. The recommended serum levels to achieve for treatment are an IC₅₀ of acyclovir at 18 µg/ml (10-15 mg/kg TID) [27]; gancyclovir at 9 µg/ml (5-10 mg/kg TID) [27, 32]. Acyclovir is a guanosine nucleoside analog that once phosphorylated by viral thymidine kinase can bind to viral polymerase, resulting in premature chain termination of viral DNA [35]. Valacyclovir is a pro-drug of acyclovir, which is converted in the body and famciclovir is the pro-drug of penciclovir [32]. Patient and physician preference dictate which drug is to be used as there is no difference in their efficacy. Treatment is recommended for at least two weeks, after which time if the patient remains asymptomatic, the drug is discontinued [32]. By the time effective drug concentrations are sufficient to inhibit virus replication at the site of entry, any virus resulting from the exposure generally has time to enter innervating neurons. Thus, the goal of prophylaxis is to minimize the amount of virus that populates the dorsal root or cranial ganglia.
The CDC recommends prophylaxis after potential B virus exposure, depending on the circumstances of the situation: if the monkey is infected, location of the injury and how quickly treatment was administered [32].

1.1.2 Immune Response to herpes viruses

The immune system is divided into two arms, the innate and adaptive immune systems. While for simplicity of understanding they are often considered separate systems, it is important to remember that the two arms communicate to help mount an effective immune response to a pathogen. Innate immune responses are relatively non-specific that involve a number of cells, some of which include monocytes, macrophages, dendritic cells, natural killer cells (NK), and neutrophils. An important consideration is that other cell types, such as cells within the mucosal and skin epithelium or non-professional cells, also initiate innate responses, such as the production of cytokines and chemokines. Importantly, these early innate responses also help to activate the adaptive arm of the immune response, including T cells and B cells for effective control of the invading pathogen.

Cells can recognize a foreign pathogen through distinct pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by pathogen specific components or through products of the damage they cause, respectively. Cells have specialized systems for the detec-
tion of PAMPs/DAMPs called pathogen recognition receptors (PRRs). PRRs include RIG-I-like receptors, toll-like receptors (TLR), NOD-like receptors, C-type lectin receptors, and the DNA sensors.

1.1.2.1 Retinoic acid-inducible gene I-like receptors (RLRs)

The retinoic acid-inducible gene I (RIG I) like receptors (RLRs) include three known cytoplasmic sensors: RIG-I [36], melanoma differentiation-associated gene 5 (MDA-5) [37] and laboratory of genetics and physiology 2 (LGP2) [38, 39], which recognize virus-derived RNA. They are adenosine-triphosphate (ATP)-dependent RNA-helicases that unwind dsRNA molecules. RIG-I recognizes short dsRNA structures and 5′triphosphate ssRNA and MDA-5 recognizes longer and more complex RNA structures [40]. HSV has been shown to activate the RLR pathway in mouse fibroblasts although the mechanism of action was unclear [41], and recent studies have implicated cross-talk with other PRRs to induce a response to HSV, and showed a requirement for HSV entry and replication [42]. Although the source of activation by dsDNA viruses is unclear with respect to cytoplasmic RNA sensors, Weber et al, have shown that after HSV infection, dsRNA does accumulate but the source of the RNA, whether cell-derived or from virus, is undetermined [43].

1.1.2.2 Toll-like receptors

The toll-like receptors (TLRs) are the best characterized PRRs and recognize a diverse array of PAMPs and DAMPs. Toll-receptors were first discovered in the *Drosophila* species and the mammalian homologs were later identified as a crucial sensor in innate immune responses and referred to as Toll-like receptors (TLRs) [44]. Currently there are 12 identified TLRs in mammals of which TLRs 1-11 form homo- or heterodimers. Upon binding by their PAMP or DAMP, TLRs initiate signaling events that lead to immune gene activation and are either MyD88-dependent (Figure 6) or TRIF-dependent (Figure 7) [45]. TLR 1 is an extracellular PRR and recognizes lipoproteins when hetero-dimerized with TLR2. Extracellular TLR2 can also form a dimer with TLR6 to sense lipoprotein PAMPs, and the DAMPS: heat shock proteins (HSPs), the non-histone chromatin associating protein high-mobility group box 1 (HMGB1), and extracel-
lular matrix (ECM). TLR3 is both extracellular and endosomal and recognizes dsRNA and mRNA. TLR4 recognizes lipopolysaccharide (LPS) and can detect a number of DAMPs, such as HMGB1, HSP, ECM, reactive oxygen species (ROS), β-defensins, amyloid B and ox-LDL. TLR5 detects bacterial flagellin. TLRs 7 and 8 form a dimer in the endosome for the detection of ssRNA. TLR9 is endosomal and detects unmethylated CpG motifs in host, viral, and bacterial DNA. Recently it is thought that TLR9 detects extracellular DNA because B cells exposed to extracellular self-DNA resulted in TLR9 activation [46]. Therefore, the route may be more important than the methylation state of the DNA. TLR 10 is non-functional in humans, at least as of now. Lastly, TLR11 detects profilin-like molecules and uropathogenic bacteria (Table 3) [45].

Compartmentalization of TLRs allows for specialized responses to pathogens. TLRs 1, 2, 4, and 5 are located on the cell surface, allowing for extracellular sensing of pathogens, and primarily detect pathogen products such as, glycoproteins or membrane structures. The TLRs 3, 7, 8 and 9 are endosomal allowing for intracellular sensing of pathogens by detection of bacterial or viral nucleic acids. Additional cell types, namely the antigen-presenting cells (APC) are capable of taking up infected or necrotic cells, and inducing endosomal immune responses to extracellular pathogens.

### Table 3 TLRs and their associated PAMPs and DAMPs

TLRs recognize a number of PAMPs and DAMPs allowing for specialized detection and subsequent immune response activation [45].

<table>
<thead>
<tr>
<th>TLR</th>
<th>PAMPs</th>
<th>DAMPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>(w/TLR2) triacyl lipoprotein</td>
<td>n.d.</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoproteins</td>
<td>(w/TLR6) HMGB1, HSPs, ECM</td>
</tr>
<tr>
<td></td>
<td>(w/TLR1) triacyl lipoprotein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(w/TLR6) diacyl lipoprotein, LTA, zymosan</td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>mRNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, viral envelop proteins</td>
<td>HMGB1, HSPs, ECM, Ox-phospholipids, β-defensin 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(w/TLR6) Amyloid-β, Ox-LDL</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>n.d.</td>
</tr>
<tr>
<td>TLR6</td>
<td>(w/TLR2) Diacyl lipoprotein, LTA, Zymosan</td>
<td>(w/TLR2) HMGB1, HSPs, ECM</td>
</tr>
<tr>
<td>mTLR5/hTLR8</td>
<td>ssRNA</td>
<td>ssRNA (immune complex)</td>
</tr>
<tr>
<td>TLR9</td>
<td>DNA, hemozoin</td>
<td>DNA (immune complex)</td>
</tr>
<tr>
<td>TLR10</td>
<td>Unknown</td>
<td>n.d.</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin-like molecule</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Uropathogenic bacteria</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
TLR signaling is initiated by binding of the respective PAMP or DAMP to the TLR dimers. Upon binding, TLRs 1, 2, 7, 8, and 9 each use the myeloid differentiation primary response protein 88 (MyD88) dependent signaling pathway (Figure 6) [45]. TLR3/4 signaling use a toll/interleukin-1 receptor (TIR) domain containing adaptor protein inducing IFN-β (TRIF or TICAM1)-dependent pathway (Figure 7) [45]. These signaling pathways may differ slightly in specialized cell types.

**Figure 6 MyD88-dependent TLR signaling pathway**

TLRs 1, 2, and 4-9 signal through a MyD88 signaling pathway. There are multiple signaling adaptors recruited upon ligand binding, and many mechanisms to limit TLR activation, both cellular and pathogen mediated. The end point of TLR activation is increased gene expression of immune factors to recruit and activate immune responses [45].

The MyD88 dependent pathway is initiated by binding of the PAMP to the TLR dimers, which then recruits the MyD88 adaptor molecule or the TIR-associated protein (TIRAP). These molecules asso-
ciates with IL-1R associated kinases (IRAKS) and tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF 6). This results in the activation of the canonical inhibitor of kappa light polypeptide gene enhancer in B cells, kinases (IKK α/β) and subsequent nuclear factor κB (NFκB) activation. The TRIF depending signaling pathway, utilized by TLR 3 and 4, is activated upon dsRNA or LPS binding leading to the recruitment of TRIF and TRIF-related adaptor molecule (TRAM), which then recruits TRAF family member associated NFκB activator (TANK)-binding kinase (TBK1) and IKKε/τ and interferon regulatory factor (IRF) 3 activation. These transcription factors then enter the nucleus to direct pro-inflammatory gene activation and Type I interferon production that direct specific immune responses to a pathogen [45].

HSV has been shown to activate a number of TLRs. HSV can activate TLR2 through unknown mechanisms in bone marrow-derived dendritic cells [47], microglia [48], Chinese hamster ovary cells [49], and HEK293 cells [48, 50] transfected with TLR2. TLR2 is also known to sense several DAMPs, some of which include HSP60 and 70 which are upregulated following HSV infection [51, 52]. HSV-2 infection leads to the production of non-histone chromatin associating protein high mobility group box 1 (HMBG1) [53], which is a known PAMP of TLR2 [54]. TLR3 has been shown to respond to dsRNA generated in the cytoplasm following HSV infection of fibroblasts, although the source of the RNA, whether viral or host, is unclear. TLR3 deficient patients have a higher prevalence of HSV encephalitis, indicating an important role for TLR3 mediated sensing in HSV infection [55]. TLR9 is a well-known sensor for HSV DNA. Plasmacytoid dendritic cells, B cells and macrophages have been shown to sense HSV DNA through TLR9 [56-59]. Although mice lacking TLR9 show a reduction in the production of Type I IFNs, virus control is similar to control mice [56]. This indicates an important, however, non-exclusive requirement for TLR9 signaling during HSV infection but appears to play a role in limiting virus spread along with TLR2 [56, 60].

1.1.2.3 NOD-like receptors

Another PRR includes the nucleotide oligomerization and binding domain (NOD)-like receptors (NLR). There are over 20 known NLR family members, although NLRP3 is the most widely studied.
Figure 7 TRIF-dependent TLR signaling pathway

TLRs 3 and 4 are the only TLRs to signal using the TRIF adaptor. TLR4 can also use MyD88. Activation of TLR3 by dsRNA leads to the recruitment of a number of cellular factors, leading to immune gene activation via NFκB and IRF 3/7. There are a number of ways to regulate TLR3 signaling, both cellular and pathogen mediated [45].

These receptors are characterized by a NOD motif, but also contain a variety of other activating domains that are crucial for function. Once they are activated, NLRs expose effector domains that can recruit signaling molecules for initiation of immune gene activation. NLRs are activated by the binding of a variety
of PAMPs: RNA or DNA; or damage associated molecular patterns (DAMPs): reactive oxygen species (ROS), cathepsin B and K+ influxes. NLR activation leads to the modulation of signaling pathways directing immune responses, such as the NFκB, MAPK, the Type I IFN pathway, and activation of the inflammasome. In the case of herpes viruses, varicella virus, which is the causative agent of chicken pox, activates NLRP3 through unknown mechanisms [61]. Additionally, HSV has been shown to activate the NLRP3 containing inflammasome at early times post-infection in human foreskin fibroblasts (HFF; Figure 8) [62].

![Figure 8 HSV-1 inflammasome activation of NLRP3 and IFI16 by HSV-1](image_url)

**Figure 8 HSV-1 inflammasome activation of NLRP3 and IFI16 by HSV-1**

HSV-1 has been shown to activate NLRP3 through unknown mechanisms, and IFI16 though nuclear and cytoplasmic dsDNA. Activation of both pathways lead to initiation of the inflammasome and IL-1β gene expression, but HSV-1 blocks mature IL-1β secretion [62].

### 1.1.2.4 DNA sensors

The last PRR to be discussed here are the recently identified DNA sensors. There are currently 10 known sensors of DNA, including DNA-dependent activator of interferon-regulatory factors (DAI or
ZBP1), RNA pol III, interferon-gamma-inducible protein 16 (IFI16), the DExD/H box helicases DDX60, DHX9, DDX41, and DHX36, Ku70, absent in melanoma 2 (AIM2) and the cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (c-GAS), to name a few.

DAI was the first cytoplasmic DNA sensor identified for HSV [63], although knockdown of DAI did not completely abrogate the response to dsDNA, indicating the presence of other sensors [64]. Recent studies indicate that HSV-2 can activate DAI in vaginal epithelial cells [65].

RNA pol III recognizes cytoplasmic dsDNA by creating RNA intermediates that can be recognized by RIG-I, at least in human 293T cells and in mouse cells [66, 67], but blocking RNA pol III did not inhibit cytokine production in human macrophages, thus the role of RNA pol III in DNA sensing is questionable [68].

The molecule IFI16 is a DNA sensor that is commonly activated following HSV infection. It can sense nuclear DNA and can shuttle between the cytoplasm and nucleus [69-78]. It has been shown to detect DNA in a number of cell types including: fibroblasts [62], epithelial cells [72], human neutrophils [77] and dendritic cells [75]. IFI16 has been shown to detect HSV DNA in HFFs [79] and monocyte-derived macrophages [76] and has been shown to induce inflammasome activation in HFFs at early times after infection, after which it is later blocked [62].

The DExD/H box helicases bind directly to DNA nucleotides and include DDX60, DHX9, DDX41, and DHX36, to name a few. These helicases signal through different mechanisms depending on the helicase involved. For example, the DHX36 and DHX9 signal through MyD88 [80], while many others signal through a complex with stimulator of interferon genes (STING) and TBK [78]. More recently another sensor of DNA has been identified as c-GAS [81, 82], which can bind directly to DNA and form a complex with STING to mediate IFN-β production.

AIM2 is a member of the PYHIN family, in which humans have 4 members, including IFI16. AIM2 binds DNA within the cytoplasm by the HIN domain independent of the sequences, and interacts with
the apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC) component to activate the inflammasome [83-85]. AIM2 has also been identified as a DNA sensor for HSV and is limited to cytoplasmic DNA [71, 84].

Ku70 is a part of the DNA-dependent protein kinase (DNA-PK) complex along with Ku80 [86], and is involved in nuclear processes such as non-homologous end-joining DNA repair, V(D)J recombination, and telomerase maintenance [87]. It has also been shown to mediate DNA sensing in the cytoplasm in HEK293T cells and mediates IFN λ production [88, 89]. Ultimately, with all of the PRRs, the goal of responding to pathogens is to mount an effective immune response to limit infection and spread and it is no different for HSV in the natural host (Figure 9).

![Figure 9 Pathogen recognition of HSV](image)

**Figure 9 Pathogen recognition of HSV**

HSV can be detected by many PRRs including TLRs 2, 3, and 9, and the DNA and RNA sensors. The pathways associated with HSV-1 recognition lead to anti-viral cytokine and chemokine expression [90].

1.1.2.5 *Innate immune cells*

Now that we understand how cells are able to sense an invading pathogen, it is important to understand the contribution of the many different cell types involved in innate immune responses and how their functions help shape an effective adaptive immune response. A few key players in innate im-
munity include the non-professional cells, such as the endothelial or epithelial cells, which are usually the primary targets of infection, and the professional cells such as dendritic cells, monocytes, macrophages, natural killer cells, and neutrophils.

In the case of HSV, the mucosal epithelium or skin epithelium is crucial in sending those first signals that something is wrong (Figure 10). Keratinocytes and fibroblasts are the skin cells used for in vitro HSV studies of primary human infection when clinical samples are unavailable. Primary cell production of cytokines and chemokines is crucial for mounting an initial response to a pathogen by recruiting and activating effector immune cells. Fluids from recurrent herpetic lesions on day 1 contained mostly IL-1β, followed by IL-6, IL-10, IL-1α and IL-12, while on day 3, contained mostly IL-6, followed by IL-1β, IL-1α, TNF-α, while IL-10 levels remained unchanged. Chemokines were also produced from recurrent lesions and contained mostly MIP-1β, followed by MIP-1α and RANTES [91]. Keratinocytes are one of the first sites of primary HSV replication, and despite rapid cell destruction after HSV infection, these were shown to be a source of IL-12, IL-10, MIP-1α, MIP-1β and RANTES on day 3 after HSV infection, and IL-1α, IL-1β and IL-6 after 6 days [91]. Additional studies showed that in vitro HSV infection of keratinocytes increased expression of the interferons α and β, TNF-α, G-CSF, GM-CSF, IL-3 and several growth factors [92], indicating an important role for these cells in sending the first signals for immune activation. Fibroblasts are the primary cells comprising the dermal layer of skin and are also a source for in vitro studies with HSV. Very little data exist on the cytokines and chemokines produced after infection of these cells. Recent studies for NLR and DNA sensor activation showed a failure of HFFs to respond to HSV with the production of IL-1β [62], but also showed an increase in IFN-β transcripts after HSV infection [79]. Additionally, recent studies in our lab showed that HFFs failed to secrete any cytokines or chemokines following HSV-1 infection including: eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, or TNF-β (M. Vasireddi et al., unpublished raw data).
Figure 10 The epidermal immune response to HSV-2

HSV-2 infection initiates in the keratinocytes which respond to virus through the production of many cytokines and chemokines, which recruit a number of innate and adaptive immune cells [93].

Following the signals received from infected primary cells, innate effector immune cells are recruited into action during infection. The first cells to enter HSV lesions appear to be neutrophils. Neutrophils, whose primary function is to destroy pathogens through the production of large amounts of ROS and cytotoxic granules within the phagosomes, are specialized innate immune cells that circulate in the blood. More recently, neutrophils have been shown to expel nuclear complexes containing DNA and granular proteins, as well as LL37 called NETs, providing another means of microbial protection [94, 95]. Neutrophils also help shape the immune response through the production of cytokines that direct antibody production by B cells [96-98], can directly activate T cells through their expression of activating receptors [99, 100] and MHC II [100] as well as transport antigen from the dermis to the lymph nodes.
Neutrophils can activate pDCs through the production of NETs [105] and coordinate with DCs to activate NK cells [106, 107]. Neutrophils have very tightly regulated systems for migration [108, 109] and the production of granules [110]. Neutrophils are well known for their ability to destroy extracellular bacteria and fungi, but more recently have been shown to play a role in anti-viral immunity to HIV [111] and poxvirus [112] through the production of NETs.

Additional cell types involved in the innate immune response to herpes viruses include macrophages, natural killer cells, dendritic cells, including myeloid dendritic cells, plasmacytoid dendritic cells, skin dendritic cells, the Langerhan cells and dermal dendritic cells. Other subsets of dendritic cells are present within lymphatic tissue are also important for immunity against herpes viruses.

Adaptive immune responses are initiated through the activation signals of innate immune cells. B cells and T cells are the most common cell types associated with adaptive immune responses. T cells can be divided into a number of subsets to include CD4+ T cells, CD8+ T cells, regulatory T cells, and suppressor T cells. The subsets of T cells can be distinguished by their transcription factor expression. Typically CD4+ T cell functions are divided into multiple subsets including two of which are the T helper-1 (TH1) or T helper-2 (TH2) responses. Their responses are called into play by the specific cytokines produced by innate cells as well as the T cells themselves, and are associated with the activation of the different subsets of T cells. TH1 is often referred to as the cell-mediated branch of adaptive immunity and usually leads to the activation of cytotoxic CD8+ T cells, which leads to cell-mediated cell death, while the TH2 refers to the antibody-mediated branch and typically leads to the activation of B cells for the production of antigen specific immunoglobulins (Igs). It is important to note that many of the other T-cell subsets likely play additional roles at different times during infection.

1.1.2.6 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells are a specialized group of lymphoid-derived dendritic cells that recognize bacterial and viral genomes through their exclusive expression of TLRs 7 and 9 [113-115].
Plasmacytoid dendritic cells are identified based on surface phenotypic markers, specifically CD4+, CD45RA+, IL3Rα (CD123)+, ILT3+, ILT1-, CD11c-, lineage-, BDCA-2, BDCA-4, CD4+, [116]. The primary function of pDCs is to produce large amounts of Type I IFNs, including 13 subtypes of IFN-α, and IFN-β, and Type III IFNs such as IFN-λ, but pDCs do not make IFN-γ, the only type II IFN. pDCs can also make tumor necrosis factor-α (TNF-α), IL-6, IL-1α/β, and several chemokines such as MIP-1 α/β, and IL-8 to recruit and activate a number of effector immune cells to the sites of infection [117-121]. Additionally, pDCs are capable of presenting antigen to T cells, although this is not their primary role. pDCs can be found in the skin, in peripheral blood, and in the lymph nodes [122].

Plasmacytoid dendritic cells were first described in the 1950s by pathological examination of lymph node and splenic tissues, as cells with a plasma cell-like morphology [123]. Decades after their initial identification, when more advanced cell immunology assays were developed, these cells were designated plasmacytoid T cells [124, 125], or plasmacytoid monocytes [126], based on the cell surface phenotypes. In the 1980’s a very similar cell-type, with plasma like morphology, was designated as the natural interferon-producing cell [127], because they produced large amounts of IFNs in response to virus infections and they were distinct from T cells, B cells, monocytes and NK cells [127-130]. Only very recently, in 1997, were these cells shown to be of dendritic cell origin and were called DC2 based on their ability to differentiate into TH2 DCs [125]. In 1999, the natural interferon-producing cell was confirmed to be the same as the plasma-like DC2 and thus designated plasmacytoid dendritic cells (pDC) [116, 126].

The pDCs exclusively express TLRs 7 and 9 to allow the detection of viral nucleic acids. A commonly used positive control for TLR9 activation is synthetic single-stranded DNA containing unmethyalted phosphorothioate-linked CpG motifs (CpG; CpG ODN) [131-134]. These TLRs are endosomal, activated in response to foreign DNA in most cases. TLRs 7 and 9 share a signaling pathway that is mediated by MyD88. Following activation, a signal transduction complex is formed in the cytoplasm and is
composed of many different proteins, including MyD88, IRAKs, TRAFs, and IRF7 [115, 135-137] (Figure 11). IRF7 is constitutively expressed in pDCs, along with IRF4, and IRF8 [138], due to lack of translational repressors (4E-BPs) [139], and upon activation is rapidly translocated to the nucleus for IFN transcription initiation when TLR signaling is initiated by the early endosome. This signaling complex is also capable of activating the NFκBs [140] and MAP kinases [141] by engagement of ligands in the late endosome to direct the activation of pro-inflammatory cytokine and chemokine genes and co-stimulatory molecule expression [134]. The constitutive expression of the IRFs allows for a rapid IFN response independent of the IFNαR feedback that most other cells require [142]. IRF 8 amplifies the IFN response during the second phase of IFN transcription and IRF 4 is a negative regulator of proinflammatory cytokine production by interacting with MyD88 [143], but does not interfere with IRF7-dependent IFN responses.

Upon activation pDCs undergo maturation in which they downregulate IFN-α and pro-inflammatory cytokine production and up-regulate co-stimulatory molecules for antigen presentation and T cell activation, such as CD40, CD80, CD86, CD83, ICOS-ligand and OX40 ligand [144]. pDCs are efficient activators of T cells [115, 122, 125, 145-148], although they are less efficient than the conventional DCs due to the short half life of MHC II on the cell surface and the rate at which they take up antigen [149]. pDCs have been shown to activate T cells through direct MHCII expression, but also through transfer of Ag to lymph node resident DCs for cross-presentation to CD8 T cells in cutaneous HSV-1 infection in mice [150].
Viral nucleic acids are taken into pDCs by endocytosis and transported to the endosomes for activation of TLR7/9. This pathway is mediated by MyD88 and triggers a signaling cascade that leads to a complex formation consisting of IRAK1, IRAK4, TRAF3/6, which activate constitutive IRF7 to induce IFN gene transcription. Additional signaling molecules can then activate NFkB's and MAPKs to induce pro-inflammatory cytokine production and co-stimulatory molecule expression [151].

pDCs respond robustly to HSV-1 through the production of Type I IFNs [41, 56, 57, 122], and thus HSV-1 is commonly used as a positive control for pDC activation and was used in the experiments presented in this dissertation. HSV contains numerous CpG motifs that can interact with TLR9 [152] to activate pDCs via TLR 9 [42, 56, 57], and independently of TLR 9 [153]. It has been shown that pDCs are important in in vivo studies as they are recruited to active HSV-2 lesions and are important for recruiting and activating T cell responses [122]. Also, antibody depletion studies in mice showed that pDCs, dispensable in controlling systemic infection, were necessary for IFN-γ production by cytotoxic T cells. Absence of pDCs extended the time for virus clearance [150]. pDC-induced IFN-α has been shown to activate HSV specific CD4+T cells in mice for the production of IFN-γ and IL-10 [154]. This IFN has also been shown to induce the migration of T cells and NK cells through the production of chemokines [58].
are also important in reducing viral titers in HSV-2 infected mice since pDC depleted mice showed worse survival than normal mice [155]. Additional studies in mice showed that pDC depletion reduced the systemic IFN-α and pro-inflammatory cytokine production, and subsequent activation of NK cells and CD8+ T cell responses to HSV-1. But for localized infections, pDCs did not reduce the viral burden or survival in subcutaneous HSV-1 or intravaginal HSV-2 infection [156]. In vitro studies of human pDCs showed that HSV-1 activated pDCs stimulated CD4 T cells to express perforin and granzyme, and produce IL-10 and IFN-γ [157]. Also, Kittan et al. reported patients suffering from HSV retinal necrosis had reduced pDC and CD8 T cell numbers [158], although Moss et al. found no correlation with circulating pDC numbers and the clinical severity of HSV-2 infection [159].

Massive production of IFNs and pro-inflammatory cytokines from pDCs helps to shape both the innate and adaptive immune responses to pathogens. pDC-induced IFN-α and TNF-α promotes autocrine survival as well as survival and maturation of bystander pDCs [154]. Cytokines from pDCs can robustly recruit and activate NK cells for lysis of infected cells [56, 58, 160, 161]. pDC induced cytokines also shape the immune response by activating monocytes to increase TLR7 expression and surface expression of pDC markers [161, 162], and can induce monocytes to produce IFN-α in response to HSV [163] as well as induce IP-10 production from monocytes to recruit other effector cells [164]. pDCs can also shape the antibody response by IFN-α induced expression of B cell activating factor (BAFF) on mDCs [165], inducing B cell differentiation into antibody secreting plasma cells through the production of IFN-α and IL-6 [166], and can induce class-switching of plasma cells [167]. Overall, the pDCs response is important for combating pathogens through the production of IFNs and cytokines and chemokines for the recruitment and activation of effector immune cells, all of which contribute to the overall host response to a pathogen.
1.1.3 Type I Interferon Pathway

Interferon was first discovered in 1957 when two researchers, Isaacs and Lindenmann, were exploring a new concept of viral interference. This was the first time a soluble factor was identified and shown to provide protection to uninfected cells [168]. The Type I Interferon pathway is important for the induction of an antiviral state within infected hosts. This pathway is initiated by a number of PRRs, such as TLRs, RLRs, DNA sensors, nucleic acid sensing molecules, such as RIGI, MDA5 for RNAs and DAI and IFIs for DNA detecting the invading pathogen, as discussed previously. Activation of the PRRs leads to the recruitment and activation of signaling molecules that are specific for antiviral genes to control the invading pathogen. The downstream activation targets differ, but ultimately activate interferon stimulated gene factors (ISGFs) that can direct interferon-stimulated gene (ISG) transcription. These genes are then transcribed and the proteins act at different stages of virus replication and gene transcription. A specific gene activated through any of these pathways is type I interferons α and β. There are five types of Type I IFNs, IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. Of these, IFN-α and β are the main subtypes of interest in antiviral immunity. There are 13 subtypes of IFN-α (α1, α2, α4, α5, α6, α7, α8, α10, α13, α14, α16, α17, and α21) in humans and only one of IFN-β [169-171]. These two IFNs bind to a common receptor, the IFNαR, which is composed of two subunits of IFNαR1 and IFNαR2. These complexes dimerize and can be bound by the different subtypes of Type I IFNs, which triggers a signaling cascade. The signals are mediated by a number of adaptor molecules, depending on the activating ligands (Figure 12) [172]. Ultimately, these pathways culminate in the activation of interferon-stimulated genes, such as IFN-α/β, pro-inflammatory cytokines, protein kinase R (PKR), MDA-5, 2′5′oligoadenylate synthase (OAS), and IRFs [173]. These gene products then act by a number of mechanisms to establish an anti-viral state within the host.
Figure 12 Signaling pathways activated by the I-IFN receptor

Signaling pathways followed by the binding of ligands for the Type I IFN receptor culminates in activation of the JAK/STAT pathway, leading to the recruitment of several adaptor molecules such as IRFs, which can then enter the nucleus to direct the transcription of many ISGs [172].

For this dissertation, the focus will be on the pathway mediated by IFN-α, or the Janus activated kinase-signal transducer and activation of transcription (JAK/STAT) pathway. IFNαR1 is constitutively associated with tyrosine kinase 2 (TYK2) and IFNαR2 is associated with JAK1 [174-176]. Upon receptor binding by IFN-α/β, JAK1 and TYK2 are transphosphorylated and then the JAKs phosphorylate the IFNαR subunits on tyrosine residues [177, 178]. These phosphate residues allows for docking by the recruited STAT molecules via the src-homology domains (SH2) [174, 179]. Depending on the signals received, the JAK1 and TYKs can recruit several different STAT molecules. There are currently 6 STAT molecules activated after Type I IFN binding, including STATs 1-6, although expression of some isoforms are limited to certain cell types [174, 179-186]. Once recruited to the receptors, STATs are phosphorylated by JAKs. Activated STAT5 can form homodimers such as STAT1, STAT3, STAT4, STAT5, and STAT6 and heterodimers of STAT-1/2, STAT1/3, STAT1/4, STAT1/5, STAT2/3, and STAT5/6 [179-181, 187, 188]. For STAT1
and STAT2, the activating phosphorylation occurs on a single tyrosine residue, at Y701 and Y690, respectively. STAT1 is also phosphorylated at Ser 727, which is required for optimal gene transcription, but not for nuclear translocation [189, 190]. Activated STAT1/2 then associates with IRF9 (ISGF3 complex). The ISGF3 enters the nucleus and binds to interferon stimulated regulatory elements (ISRE) on DNA to direct ISG transcription (Figure 13) [174, 179-181, 185, 191]. Within the nucleus STATs interact with a series of co-activators such as p300 and cAMP-responsive element binding proteins (CREB) binding protein (CBP), which help to regulate chromatin remodeling for access to promoters of ISGs [192-194].

**Figure 13 Classical JAK-STAT signaling pathway**
The binding of IFN-α or β activates the JAK-STAT signaling pathway, leading to the activation and recruitment of STAT homo- or heterodimers that complex with IRF9 and enter the nucleus to bind to ISRE direct ISG transcription [194].

Herpesviruses are known to block many steps of the Type I IFN pathway. HSV can block the induction of the IFN pathway by blocking protein kinase R (PKR) activation by US11 late in infection, an RNA binding protein [195-199], or by γ34.5 early in infection forming a complex with protein phosphatase 1 (PP1) to reverse PKR activation [196, 200, 201]. HSV also interferes with the IFN signaling pathway by blocking the activation of JAK via ICP0 [201, 202], and prevents the nuclear translocation of STAT by
ICP27 [203]. Furthermore, US3 hyperphosphorylation of IRF3 prevents nuclear translocation [204], and blocks ISG mRNA induction [205], although this has recently been challenged [206]. Very recently, VP-16 has been shown to interfere with IRF-3 mediated gene activation by disrupting the interaction of IRF3 and the co-activator, CREB-binding protein (CBP) [207]. HSV can also block the signaling pathways induced by secreted IFNs, such as preventing the phosphorylation of JAK/STATs in the IFN signaling pathway, although this is dependent on cell type [208] as well as inducing the suppressor of cytokine signaling (SOCS), which binds to JAKs to block their activation [209] to limit ISG activation against HSV.

1.2 Research Goals

The goals of this dissertation were to explore the interferon response within the context of the foreign and natural hosts to determine whether this response may correlate with the pathogenesis of infection, or plainly said, why macaques live, having a robust immune response and humans die with little-to-no defense responses. Because the interferon pathway is so crucial to the development of an antiviral state, either as a direct effect on infected cells or through indirect activation of effector functions of immune cells, I aimed to characterize this response in both hosts. This dissertation was designed to test the hypothesis that B virus modulates the IFN response during zoonotic infection by blocking pDC activation and subsequent IFN signaling pathways to circumvent host defenses, while these pathways remain intact in the macaque hosts. To test this hypothesis, I first looked at the plasmacytoid dendritic cell response within the human host using a cell culture model system to mimic events that occur when B virus infects skin or mucosal cells at the site of entry. These are one of the primary target cells crucial for initiating the interferon response as well as for helping to shape the development of adaptive immunity, both of which appear to be dysfunctional during human B virus infection. Next, I explored the macaque immune response to B virus infection ex vivo to determine PBMC response to B virus, as it was not possible to isolate pDCs from macaques due to lack of specific reagents to enrich these
cells. Further, we studied the interferon-signaling pathway within fibroblasts, from both humans and macaques, to identify differences between the two host cell responses at the primary sites of infection.
2 MATERIALS AND METHODS

2.1 Cells and Viruses

B virus stock (strain E2490; passage 74; originally a gift from E. Hull) was generated by passage in Vero E6 cells (lot CCL-81; ATCC, Manassas, VA) in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Inc; Manassas, VA) supplemented with 2% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Virus stocks were obtained by one round of freeze/thawing at -80°C and were clarified by centrifugation at 1000 rpm for 10 min at room temperature. Virus titers were determined by standard plaque assay. Virus stocks were kept at -80°C until use. All experiments conducted for this dissertation were done using the same stock of the P74 strain of B virus E2490. All experiments with B virus were done in a CDC-Registered BSL-4 laboratory in compliance with the Department of Homeland Security’s regulations for Select Agents, in effect during most of this project.

Herpes simplex virus-1 (strain MacIntyre; passage 7; lot VR-539; ATCC, Manassas, VA) was generated by passage in Vero E6 cells (lot CCL-81; ATCC, Manassas, VA) in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Inc; Manassas, VA) supplemented with 2% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Virus stocks were obtained by one round of freeze/thawing at -80°C and were clarified by centrifugation at 1000 rpm for 10 min at room temperature. Virus titers were determined by standard plaque assay. Virus stocks were kept at -80°C until use.

2.2 Virus Stocks and Plaque Assay

Virus stocks were generated in Vero E6 cells in confluent roller bottles. Cells were infected at an MOI of 0.01 for 48 h. For collection, the roller bottle was shaken or scraped to dislodge any remaining cells then transferred to a 50 ml conical tube and centrifuged for 10 min at 1000 rpm. Supernatants were discarded and the pellet was resuspended in 5 ml of diH2O with 2X protease inhibitor. Virus stocks were frozen and thawed once prior to aliquoting. Virus stocks were stored at -80°C until needed.
Virus titers were determined by standard plaque assay. Briefly, Vero E6 cells were seeded into 12-well plates so that they were 90% confluent on the day of the experiment. Ten-fold serial dilutions were made in plain DMEM and 100 μl of each dilution was added to the respective wells. Virus was allowed to adsorb for 1 h at 37°C at which time the lysate was removed and 1 ml of 0.8% methylcellulose was added to each well. Cells were cultured for 48 h at 37°C. For collection, wells were washed with PBS until all the methylcellulose was removed and 100% methanol was added to each well for 15 min. Plates were dunked out, rinsed and staining with 0.1% crystal violet. Plaques were counted and final titers were determined.

2.3 Rhesus Macaque Fibroblast Isolation and Maintenance

Rhesus macaque fibroblasts were isolated from whole skin biopsies (Yerkes National Primate Center, Atlanta, GA). The majority of skin was obtained from the back of female macaques that were euthanized for other purposes and tissues were kindly provided through the tissue sharing program by the PI using the macaque for biomedical research. Foreskin was also obtained from male macaques, but the tissue was difficult to use due to contamination. The macaques were negative for SIV and B virus antibody.

Whole skin biopsies were placed in PBS and stored at 4°C until cells could be isolated (no more than 24 h). The tissue was shaved to remove hair and cut into small pieces with a sterile scalpel. Throughout the process the tissue was periodically rinsed in PBS containing penicillin, streptomycin with anti-mycotic. The tissue was place in 0.4% Trypsin/EDTA (Mediatech, Manassas, VA) for 30 min at 37°C with vortexing intermittently. The tissue was further cut into smaller pieces and fresh trypsin was added and kept overnight at 4°C. The following day the tissue was resuspended with vortexing and pipetting through a 25 ml down to a 10 ml. Once the majority of the tissue was disassociated, the cells were filtered using microfilters of 100 nm. The Rhesus Macaque Fibroblasts (RMF) were pelleted at 800 rpm and resuspended in DMEM with 20% FBS and 1% antibiotic/antimycotic. Depending on the cell numb-
bers, RMFs were seeded into multi-well plates or a T25 flask. RMFs were cultured until they adhered (3-4 days) with intermittent addition of fresh medium. These were cultured until confluent and transferred to the next largest growth flask at a 1:2 split ratio. This was considered passage (P) two. RMFs were grown until a consistent split at a ratio of 1:2 was confluent in two days, which varied for each animal, but was achieved by approximately P5. RMFs were used immediately or frozen in 10% DMSO with culture medium and stored in liquid nitrogen. For thawing of RMFs, tubes were placed in 37°C for rapid thaw and transferred to warm medium in a multi-well plate or T-25 at a high seeding density. RMFs were cultured for one to two passages before using for experiments. RMF purity and isolation was verified using immunofluorescence with fibroblasts markers vimentin and fibronectin and cytokeratin, a marker for keratinocyte contamination. Cells were fixed with methanol for 15 min, and blocked with normal goat serum. These were subsequently labeled with the primary antibodies followed by goat anti-rabbit or mouse IgG (Sigma Aldrich, St. Louis, MO)-conjugated to the specific fluorophores, followed by nuclear staining with Hoescht (Invitrogen, Grand Island, NY). Cells were visualized on a Zeiss AxioVert 200 (Zeiss, Thornwood, NY) (Figure 31).

2.4 B Virus Kinetics in Fibroblasts

B virus titers were determined in human foreskin fibroblasts and RMFs. Fibroblasts were grown to confluency in 12-well plates. Fibroblasts were infected (MOI 5) for 1 h at 37°C, 5% CO₂. Samples were collected at 1, 4, and 24h after infection by scraping and placed into a 1.5 ml microcentrifuge tube and stored at -80°C. Virus was quantified using a standard plaque assay (Figure 32). Additionally, to determine the amount of virus that remains cells associated, virus titers (infectious virions) were determined for cells and supernatants (sup+cells) and supernatants only (sup) by standard plaque assay (Figure 22).
2.5 Human and Macaque PBMC Isolation

Whole blood was collected from multiple healthy volunteers by vein puncture into Becton Dickinson (BD) Vacutainer tubes containing sodium heparin (BD, Franklin Lakes, NJ). Blood was collected with informed consent and with an approved IRB in accordance with federal and institutional policies. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Axis-Shield, Oslo, Norway) density centrifugation. Macaque blood was obtained from Yerkes National Primate Research Center (Grant No. RR000165) and was collected in Becton Dickinson (BD) Vacutainer tubes containing sodium heparin (BD, Franklin Lakes, NJ) from animals of varying ages prior to necropsy in accordance with Yerkes National Primate Research Center’s IACUC requirements. PBMCs were used immediately or frozen in fetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO) in cryo-containers at -80°C and stored in liquid nitrogen.

2.6 Plasmacytoid Dendritic Cell Isolation

Plasmacytoid dendritic cells were isolated from PBMCs by negative selection using the Human Plasmacytoid Dendritic Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC). Briefly, unwanted cells were labeled with tetrameric antibody complexes that recognize all non-pDCs and were selected for using dextran-coated magnetic particles. The purity of isolated pDC was assessed by flow cytometry using antibodies against BDCA-2 (CD303; Miltenyi Biotec, Auburn, CA) and BDCA-4 (CD304; Miltenyi Biotec, Auburn, CA).

2.7 Western Blot Analysis

Western blot was used to analyze protein expression in cell lysates. Briefly, cells were treated according to the experiment and cells were lysed using Laemmli buffer (Biorad, Hercules, CA) containing β-mercaptoethanol. The collection volume depended on the experiment, but ranged from 50 μl for small plates up to 500 μl for T25. Cell lysates were boiled at 99°C for 5 min, placed on ice to cool, and then
used immediately or stored at -80°C. For most samples, a 7.5% SDS-polyacrylamide gel was assembled and a 5% stacking gel. Samples were loaded in 10 μl volume and a standard marker was used. Gels were electrophoresed in running buffer for approximately two hours at 70V. Gels were transferred subsequently onto 0.45um nitrocellulose membranes (GE Healthcare Lifesciences, Piscataway, NJ) in transfer buffer either overnight at 23 V or for 1 h at 70V. Protein transfer was verified using Ponceau S. Blots were blocked in 5% blotto with non-fat dry milk for 1 h at room temperature. Primary and secondary antibodies were used according to the manufacturers instructions. Blots were visualized by addition of ECL substrate solution (GE Healthcare Lifesciences, Piscataway, NJ) at 1:1 concentration for 5 min followed by exposing to film for varying times, and developed using a Kodak Series 2000A Processor (Ti-Ba, Inc., Rochester, NY).

2.8 ELISA for Interferon-α

Interferon-α concentrations were determined using an ELISA kit for Interferon-α (MabTech, Inc., Mariemont, OH) according to the manufacturer instructions. Briefly, a 96-well high binding EIA/RIA ELISA plate (Santa Cruz, Dallas, TX) was coated with Mt 1/3/5 antibodies at 4 ug/ml in 100 ul in phosphate buffered saline (PBS; MediaTech, Manassas, VA) overnight at 4°C. Primary antibodies were removed, plates were washed and after blocking, standard dilutions of IFN-α and the samples were loaded into the wells and incubated for 2 h at room temperature. The secondary antibodies, Mt 2/4/6 were added and incubated at room temperature for 1 h, followed by the addition of strepavidin and 3, 3’, 5, 5’-tetramethylbenzidine substrate (TMB; Biolegend, San Diego, CA). The reaction was stopped with 2N H2SO4 and the sample absorbance was read at A450nm using a BioTek Powerwave HT (Winooski, VT). A standard curve was generated using GraphPad Prism™ and the unknown sample concentrations were determined.
2.9 PBMC Response to B Virus

To determine how PBMCs responded to B virus, human PBMCs were isolated from whole blood. In 96-well plates, 1x10^6 PBMCs/well were exposed to various MOIs of B virus and cultured for 18-24 h in RPMI-1640 containing 10% FBS and 1% antibiotic. Cell-free supernatants were collected by centrifuging PBMCs at 100xg for 10min. Supernatants were collected in 1% Tween/DOC and stored at -80°C until use. Additionally, PBMCs were exposed to B virus infected fibroblasts in co-cultures. Briefly, fibroblasts were infected with B virus for 1 h, 37°C, 5% CO2. PBMCs were added to the infected fibroblasts in equivalent amounts and cultured for an additional 23 h. Cell free supernatants were collected by centrifuging at 100xg for 10 min in 1% Tween/DOC and stored at -80°C until analyzed.

2.10 Luminex Assay

Supernatants from co-cultures of pDCs with infected HFFs and pDCs were used in a LiquipChip apparatus (Qiagen, Valencia, CA) for the detection of cytokines and chemokines. The kit used for analysis was the Milliplex™ Map Kit Human Cytokine/Chemokine 26-Plex (EMD Millipore, Billerica, MA) which detects eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and TNF-β from each well. The samples were analyzed according to the manufacturers instructions. The principle of the kit is the use of microspheres that contain a fluorescent dye. Based on different concentrations of these dyes the machine can detect multiple analytes from the same well. These microspheres are coated with capture antibodies specific for each cytokine and chemokine. A secondary biotinylated antibody is added to the wells and streptavidin (SA)-PE is used as a reporter molecule. The machine collects the samples and analyzes each microsphere and quantifies the fluorescence for the identification of each analyte, followed by a secondary laser excitation of PE for quantification of the analyte. Standard curves and unknown concentrations were generated and interpolated using Milliplex Analyst software (EMD Millipore, Billerica, MA).
2.11 B Virus Titer Reduction Assay

To determine if B virus was sensitive to the effects of IFN-α, either from PBMCs, PBMC co-cultures or from recombinant IFN-α, we set up several experiments. First, we tested the sensitivity of B virus titers on cultures with supernatants from B virus-exposed PBMCs. To test this, PBMCs were cultured in 96-well round-bottomed plates and exposed to B virus at an MOI 5 for 24 h. Cell-free supernatants were collected by centrifugation at 100xg for 10 min. Supernatants were then transferred to B virus infected fibroblasts at 1 hpi and cultured for 24 h. Well contents were scraped and stored at -80°C until titered by standard plaque assay.

Secondly, we determined if B virus replication was sensitive to IFN-α from co-cultures with PBMCs. To test this, fibroblasts were infected with B virus at an MOI 5 for 1 h, in 48-well plates, at which time, PBMCs were added to infected fibroblasts and cultured for 23 h. The well contents were collected by scraping and stored at -80°C. Samples were titered by standard plaque assay.

Lastly, the sensitivity of B virus was examined when fibroblasts were pre-treated for 30 min with Universal Type I IFN (PBL Interferon Source, Piscataway, NJ) at varying doses. Fibroblasts were infected with B virus for 1 h at which time IFN was added back to the cells, and cultured for an additional 23 h. Samples were collected by scraping and stored at -80°C. Samples were titered by standard plaque assay.

2.12 Plasmacytoid Dendritic Cells Response to B virus

For determination of the cytokines and chemokines produced, plasmacytoid DCs were equally distributed into a round-bottomed 96-well plates. Each treatment was added in 200 ul RPMI-1640 with 10% FBS and 1% penicillin/streptomycin. As negative controls, “untreated” consisted of medium only and mock-infected cell lysate (MCL) was added in the equivalent amount as B virus. For a positive control HSV-1 was used at an equivalent MOI as B virus, or CpG 2216 was used at 5 µg/ml or 10µg/ml (Invivogen, San Diego, CA). Treatments were added and cells were cultured for 24 h at 37°C, 5% CO₂. Cell-free supernatants were collected by centrifugation for 10 min at 100xg. Supernatants were transferred
to a clean tube containing 10% Tween/DOC for a final concentration of 1% and stored at -80°C until used. Similarly, cytokines and chemokines were measured from B virus infected HFFs co-cultured with pDCs. Briefly, HFFs were infected with an MOI 5 of B virus for 6 h then pDCs were added, and cultured for an additional 18 h. Cell-free supernatants were collected by centrifugation at 100xg in 1% Tween/DOC and stored at -80°C.

To determine if the dose of B virus affects the amount of IFN-α produced, pDCs were cultured in a 96-well round-bottomed plate and exposed to increasing MOIs of B virus for 24 h. As a positive control for cell function, CpG 2216 was used at 5 μg/ml (Invivogen, San Diego, CA). Cell-free supernatants were collected by centrifugation at 100xg in 1% Tween/DOC, and stored at -80°C until analyzed by ELISA.

2.13 B virus modulation of IFN signaling pathway

To determine if B virus modulates the IFN signaling pathway, fibroblasts were grown in T25 flasks, infected with an MOI 5 of B virus and cultured for 24h. Cell lysates were collected at 2, 4, 6, and 24 h in 500 ul Laemmli buffer, boiled for 5 min at 99°C and stored at -80°C until used. Samples were run on a 7.5% polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane. Blots were blocked and probed with an antibody against phosphorylated (Y701) STAT-1 (p-STAT-1; BD Transduction Laboratories, San Jose, CA) at 1:1000 and total-STAT-1 (BD Transduction Laboratories, San Jose, CA) at 1:1000. As a loading control, blots were probed with anti-GAPDH (Cell Signaling, Danvers, MA) at 1:1000.

Secondly, to examine if B virus blocks the IFN signaling pathway, fibroblasts were infected with B virus at an MOI 5 for 1 h, then treated with 50 U/ml of recombinant Type I IFN for 30 min. Cell lysates were collected in 50 ul of Laemmli buffer, boiled at 99°C for 5 min and stored at -80°C until used. Samples were run on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane, and probed for p-STAT-1, total-STAT-1 and GAPDH, all at 1:1000.
2.14 Plasmacytoid dendritic cells do not support B virus replication

B virus infection of plasmacytoid dendritic cells

Plasmacytoid dendritic cells were infected with B virus at 10 pfu/cell. Virus was allowed to adsorb for 1 h or left on the cells for 24 h in RPMI-1640 (Mediatech, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Norcross, GA) and penicillin-streptomycin (Mediatech, Manassas, VA). In some cases, a 50 mM citrate wash was used to inactivate any remaining virus outside of the cells. The infections of pDC were measured by standard plaque assay and quantitative PCR (see below).

PCR for B virus transcripts

Quantitative real-time PCR. DNA was isolated from B virus exposed pDCs using the Easy DNA Kit (Invitrogen, Grand Island, NY) according to the manufacturers instructions. Herpes B virus DNA copies were quantified using the Topo-PC DNA vector (Invitrogen, Grand Island, NY) containing the B virus gene for glycoprotein B (gB; Irina Patrusheva, Viral Immunology Center, Atlanta, GA). Targeted gB was amplified using the following primers for B virus gB: Forward: 5’AGGTTGATGAACGTGCTGAC 3’ Reverse: 5’CGGGCTACGTGTACTTTGAG 3’. The sequence of the gB specific internal probe containing FAM-TAMRA was 5’ ACTCCCACCAGCTGGGTCGC 3’. The PCR was performed using an ABI Prism 7900HT (Applied Biosystems, Grand Island, NY). Amplification conditions were 40 cycles of: 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min. Standard dilutions of the gB vector were generated and unknown samples were interpolated from the standard curve.

Reverse transcriptase PCR. pDCs were exposed to B virus at 2 pfu/cell and cultured for 24 h at 37°C, 5% CO2. Total RNA was purified using the manufacturer’s protocol for the TRI reagent RT (Molecular Research Center, Cincinnati, OH). To ensure DNA and RNAase removal, a DNA-free protocol (Ambion, Grand Island, NY) was implemented. For PCR of B virus transcripts the following primers (Sigma-Aldrich, St. Louis, MO) were used:

ICP0: Forward: 5’-CTCGAGCGTGGTGCCAT-3’ Reverse: 3’-GGGTCACTCCAGAGGCCGT-5’;
Thymidine kinase: Forward: 5’-TGGACCTGGCCATGCTGTC-3’ Reverse: 3’-CAGCAGTGCGACCCAGTCCAC-5’; Glycoprotein C: Forward: 5’-GGTGGAGCTGCGTGGCTG-3’ Reverse: 3’-CACTCAGCGCGGTTGCTA-5’ and \( \beta \)-actin: Forward: 5’-GCCATGTACGTTGCTATCCA-3’ Reverse: 3’-TGGATGCCACAGGACTCCAT-5’ with the Superscript One Step RT-PCR system with Platinum Taq DNA Polymerase (Invitrogen, Grand Island, NY) on a Gene Amp PCR System 9700 (Applied Biosystems, Invitrogen, Grand Island, NY) with the following program: 1 cycle at 55°C for 30 min for reverse transcription, followed by denaturation for 2 min at 94°C, and amplification with 40 cycles of 15 sec at 94°C, 30 sec at 60°C and 1 min at 68°C, followed by a final extension step at 68°C for 5 min and then held at 4°C. The amplification products were separated on a 1.2% agarose gel (BioRad, Hercules, CA) and visualized.

2.15 Statistical Analysis

Statistical significance was measured using GraphPad Prism\textsuperscript{TM} (LaJolla, CA) using a two-tailed paired student t-test, which compares the means between repeats of experiments containing one dependent variable and a minimum of two independent variables. The experiments measuring B virus titers in fibroblasts (Figures 18, 22 and 23) and in pDCs (Figure 20) were analyzed by Student’s t-test.

Analysis of variance (ANOVA) analyses were used to measure the significance of the results from experiments with two or more independent variables (Figures 16, 17, 19, and 21). ANOVA analyses require at least 3 repeats and more than two independent variables. ANOVA compares the overall mean across the independent variables while the application of a post-hoc test generates a p-value based on the means between the groups. For all ANOVA analyses a post-hoc test of Newman-Keuls was applied, which allows comparison of the mean of each independent variable with the controls, generating a p-value for each independent variable. All significant values were p<0.0001 (*** or p<0.05(**). Values that were not significant were designated with a NS.
3 RESULTS AND DISCUSSION

Based on the characteristics of zoonotic B virus infection in humans, i.e., few-to-no adaptive defenses, we designed experiments to study deficiencies in innate immunity that may underlie the failure of mounting effective adaptive defenses. We elected to study pDC responses to B virus because pDCs are the primary source of abundant amounts of Type I interferons, the primary antiviral molecules in mammalian species. There are no animal models that recapitulate infection in either macaque or humans therefore; we designed a cell culture model system to better understand innate defenses following B virus infection. To utilize this model, we first collected fresh, whole peripheral blood from multiple healthy humans and isolated PBMCs by density centrifugation. It is important to point out that B virus is not viremic (detected in blood) and the additional of peripheral blood PBMCs was to mimic the infiltrating PBMCs from blood into infected tissues. We then used a pDC isolation kit to purify pDCs from pooled PBMCs by negative selection, which prevented receptor-mediated activation and led to a 97% enrichment of pDCs that were BDCA-2 (CD303) and BDCA-4 (CD304) positive (Figure 14). There are currently no available reagents for the isolation of pDCs from macaques so we used human pDC and human/macaque PBMC responses following exposure to B virus as a measure of species-specific IFN responses.

3.1 Human plasmacytoid dendritic cell response to B virus

Both natural and zoonotic B virus infections usually originate in the skin through a bite or a scratch from an infected monkey or a splash with contaminated fluids into an open mucosal site (e.g., eye, mouth, etc.) or external wound. The virus replicates at the site of entry within keratinocytes and fibroblasts, the first target cells infected. These infected cells, and possibly adjacent cells often send signals in the form of cytokines or chemokines to recruit nearby immune cells, such as plasmacytoid dendritic cells unless these host defenses are blocked by the virus. Plasmacytoid dendritic cells are important producers of Type I IFNs that engage innate defense activation and shape type of adaptive im-
mune pathways engaged as a result of viral infections. Dendritic cells are central for the generation of an immune response. A characteristic of zoonotic B virus infection, which is the induction of limited, early and ineffective adaptive defenses, along with the knowledge of the roles of pDCs in antiviral immunity, led us to formulate the hypothesis that B virus blocks the production of cytokines and chemokines from human plasmacytoid dendritic cells (pDC). We tested our hypothesis in a human cell-culture model to mimic natural infection (Figure 15). We quantified pDC cytokine and chemokine production following pDC exposure to B virus infected cells through co-culture with these versus direct exposure to B virus released from infected cell lysates. B virus replication in human fibroblasts results in the fusion of infected and healthy fibroblasts, syncytial formation, to ultimately limit the release of virions, potentially limiting the numbers of virions to which defense systems are exposed.

The pDCs were isolated by negative selection from PBMCs from healthy individuals who were sero-negative to B virus, HSV-1 and 2, using the Human Plasmacytoid Dendritic Cell Isolation Kit (Stem Cell Technologies, Vancouver, BC). The isolation yielded pDCs with a purity of approximately 98% as measured by FACS screening for BDCA-2 and BDCA-4 positive cells (Figure 14). Due to the low concentration of pDCs in peripheral blood (0.1-0.8% of PBMCs) it was necessary to pool blood from multiple donors. Although pDCs could be recruited to infected lesions from blood, our experimental setup utilized peripheral blood pDCs representative of infiltrating pDCs and we used pDCs numbers much higher (10³-10⁴ pDCs/experiment) than would be observed in natural infection (~1-2 pDCs per lesion) because the activity of pDCs in tissue would be below detection. We have previously shown that pDCs from different donors responded differently to synthetic DNA (CpG/ODN 2216) (Figure 30 in Appendix). To correct for differential responses between heterogeneous pDCs, we used two negative controls: (i) pDCs exposed only to culture medium to determine any basal pDC responses and (ii) pDCs exposed to uninfected cell lysates, which were generated in the same way as B virus stocks, designated as mock cell lysate (MCL) and at least one positive control (i) HSV-1, endemic in humans, was used because it induces
high amounts of IFN-α from pDCs and is a closely related herpes virus to B virus, and/or (ii) CpG/ODN 2216, which is a synthetic DNA oligonucleotide that induces IFN-α via TLR9.

Figure 14 Human pDC purity after isolation from PBMCs.
BDCA-2 and-4 were used as markers of pDCs within PBMCs (A) and after pDCs purification using the Stem Cell Human pDC Isolation Kit (B), which resulted in a purity of approximately 98% (bottom right) by FACS analysis. PBMCs contain approximately 1.5% pDCs prior to isolation. As a negative control, cells were left unlabeled (left squares) to rule out auto-fluorescence. Double-labeled pDCs are shown in the right column and are positive for BDCA-4 and BDCA-2.
Figure 15 The experimental design to measure the pDC response to B virus

A. Illustrates the co-culture system of pDCs with B virus infected human foreskin fibroblasts (HFFs). HFFs (2x10^4) were exposed to two negative control groups (1) Medium and (2) MCL. For the test group, HFFs were infected with (3) B virus (MOI 5) and for a positive control (4) HSV-1 (MOI 5). All treatments were kept on HFFs for 1h, at which time the treatments were removed and fresh culture medium added. At 6hpi, 1x10^4 ppDCs were added to treated HFFs and cultured an additional 18h. Supernatants were collected and analyzed for 26 different cytokines and chemokines.

B. 1x10^4 pDCs were exposed to one of four treatments to determine how pDCs respond when directly exposed to B virus cell lysates. As negative controls, pDCs were exposed only to (1) culture medium and or to (2) MCL (mock cell lysate) which was added at the equivalent amount as for the B virus treated group. The test group consisted of pDCs exposed to (3) B virus (MOI 5) and as a positive control pDCs were exposed to (4) HSV-1 (MOI 5).

Our first goal was to test the hypothesis that **pDCs would not produce cytokines or chemokines after exposure to B virus via infected human foreskin fibroblasts (HFFs).** Our goal was to measure the response of pDCs exposed to B virus infected HFFs. A pictorial of this experiment is presented in Figure 15A. To verify that pDCs were capable of producing cytokines and chemokines, we cultured pDCs directly with B virus released from infected cell lysates and the controls as depicted in Figure 15 B. Human foreskin fibroblasts (HFFs) were grown to near confluence (2x10^4 HFFs/well) in a 96-well plate and exposed to one of the four different treatments: (1) culture medium only, (2) MCL, (3) B virus (MOI 5), and HSV-1 (MOI 5). To evaluate that a measureable response was due to B virus, we used two negative controls, medium and MCL, present in the same amounts found in B virus lysates. HSV-1 was the positive control because it is a closely related herpes virus endemic in humans and is known to induce robust amounts of IFN-α from pDCs, as previously discussed. Virus was adsorbed to cells in minimal amounts of
medium for 1 h at 37°C. Unadsorbed virus, medium, and MCL were removed from each of the respective wells in this experiment and replaced with pDC culture medium (RPMI-1640 supplemented with 10% FBS and 1% antibiotic). The HFFs were cultured for a total of 5 h after adsorption to allow B virus to progress through the virus replication cycle. By this time, immediate-early, early, and some late genes have been expressed. At 6 hpi, the culture medium was removed and 1x10^6 freshly enriched pDCs were added in fresh medium and this co-culture was incubated for an additional 18 h. In parallel, the aliquots of this pool of pDCs were seeded into separate 96-well plates to measure their response to B virus released into infected cell lysates. Controls were also exposed to the same control treatments used for the parallel experiment. The supernatants were collected 18 h after the addition of pDCs and clarified by centrifugation at 100xg for 10 min. Each supernatant contained tween/DOC, at a final concentration of 1% to ensure that any B virus present was inactivated prior to removal from the BSL-4, in accordance with the CDC’s BMBL guidelines (5th Ed.) The supernatants were removed from the BSL-4 and stored at -80°C.

To maximize the amount of data collected from these samples, a multiplex assay was used to measure the pDC response to (1) B virus infected HFF in co-cultures and (2) B virus released into infected lysates. The Milliplex™ Map Kit Human Cytokine/Chemokine was purchased from Millipore to quantifies the levels of 26 cytokines and chemokines within each collected sample, viz., eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and TNF-β. The principle of the kit is the use of microspheres that contain a fluorescent dye. Based on different concentrations of these dyes the Luminex machine can detect and differentiate multiple analytes from the same well. These microspheres are coated with capture antibodies specific for each cytokine and chemokine. A secondary biotinylated antibody is added to the wells and streptavidin (SA)- phycoerythrin (PE) is used as a reporter molecule. The machine collects the samples and analyzes each microsphere and quantifies the fluorescence for the
identification of each analyte, followed by a secondary laser excitation of PE for quantification of the analyte. The software supplied by Millipore was used to determine the concentration of each analyte based on a standard curve generated from known standards. As shown in Figure 16, pDCs co-cultured with B virus infected HFFs (white bars), produced significant amounts of IFN-α and TNF-α, but none of the other cytokines measured. In contrast, pDCs exposed to B virus released into infected cell lysates (gray bars) produced significant amounts of IL-1α, IFN-α, IL-6, and TNF-α, possibly as a result of exposure to unpackaged viral DNA also released into lysates. Similarly and as shown in Figure 17, pDCs co-cultured with B virus infected HFFs produced significant levels of IP-10, whereas, pDCs directly exposed to B virus infected cell lysates responded with significant production of IL-8, MIP-1α, MIP-1β and IP-10. The raw values for the cytokines and chemokines are presented for inspection in Tables 4 and 5 located in the Appendix. To better understand the impact of pDCs exposed during in zoonotic infection, it is important to consider the functions of the pDC produced cytokines and chemokines in relation to the recruited immune cells and the potential consequences.

IL-1α is also known as fibroblast-activating factor and B cell-activating factor. It is constitutively produced by epithelial cells, and can be induced in a number of cells, including fibroblasts, macrophages, granulocytes and dendritic cells, just to name a few. IL-1α has been shown to activate keratinocytes and macrophages to induce more IL-1α, but also can enhance the proliferation of fibroblasts, CD4+T cells, induce the production of IL-2 and IL-6, activate CD8+ T cells, induce the proliferation of mature B cells and induce Ig production in the presence of mitogens. As shown in the top panel of Figure 17, pDCs co-cultured with B virus infected fibroblasts failed to induce any significant amount of IL-1α (1.75 pg/ml), while direct exposure to B virus and the contents of lysed infected cells induced ~100pg/ml of IL-1α, which was significant by ANOVA with a post-hoc analysis of Newman-Keuls. Our positive control, HSV-1 infected cells co-cultured with pDCs failed to result in the production of significant levels of IL-1α, whereas exposure of pDCs to HSV-1 released into infected cell lysates induced 2.5 times greater levels of
IL-1α than pDCs directly exposed to B virus released into infected cell lysates. Each of the negative controls of medium and MCL failed to induce any IL-1α from pDCs exposed to lysed cells or co-cultured with HFFs, underscoring that the response was specific to virus and not cells, intact or lysed. These data suggest that B virus presented within the context of the co-culture experiments does not induce measurable IL-1α production from pDCs. IL-1α production during zoonotic infection could support multiple aspects of the immune response to B virus by activating and inducing the proliferation of T cells and B cells, neither events observed in B virus infected humans. The lack of production of IL-1α from pDCs in response to B virus infected HFFs implies that pDCs would fail to activate T and B cells or support the healing of skin at the site of infection. The positive control, HSV-1 infected fibroblasts, also failed to induce any IL-1α from pDCs, indicating that IL-1α may not be an important player in simplex virus infections.

The presence of IFN-α can influence both innate and adaptive immune cells, including NK cells, B cells, T cells, monocytes and dendritic cells, but can also affect primary cells, such as fibroblasts that are permissive to infection by B virus. IFN-α can turn on various and multiple anti-viral IFN-dependent gene pathways such as RIG-I, and MDA-5, NF-κB, and induce the expression of interferon-stimulated genes (ISGs). pDCs provide a major source of IFN-α in the context of herpes virus infections. As shown in the second panel of Figure 16, B virus infected HFFs co-cultured with pDCs (white bars) produced significant levels of IFN-α at 300pg/ml, while pDCs exposed to B virus release in infected cell lysates (gray bars) produced 2500 pg/ml of IFN-α, 8 times more than in co-cultures. In data not shown, HFFs alone failed to induce any IFN-α after B virus infection (N. Brock, 2013 and M. Vasireddi, 2009), underscoring that pDCs are a source of IFN-α. Our negative controls of medium and MCL, failed to induce significant amounts of IFN-α (4 pg/ml and (14.23 pg/ml), respectively) from co-cultures of HFFs with pDCs, indicating that the observed responses were not attributable to cells or cellular organelles. Our positive control of HSV-1 infected HFFs co-cultured with pDCs (white bars) resulted in the production of 450 pg/ml of IFN-α while
pDCs exposed to HSV-1 released into infected cell lysates (gray bars) produced 5000 pg/ml of IFN-α from pDCs, both levels which were significant relative to levels produced as a result of just co-culture or lysed cells when using ANOVA. The impact of IFN-α produced from pDCs was explored in this dissertation in the context of Type I IFN signaling pathway activation and will be discussed later (Figures 25-28) and the effects of IFN-α on the extent of B virus replication in fibroblasts (Figures 23 and 24). The extent of the reach and activity of the secreted IFN-α needs further investigation in the context of immune cell activation.

IL-6 is a cytokine known to induce B cell differentiation into plasma cells, supports T cell growth and differentiation, induces the development of macrophages, activates keratinocytes and mediates lymphocyte trafficking. As shown in the third panel of Figure 16, IL-6 (400 pg/ml) was produced by pDCs co-cultured with HFFs exposed only to medium while MCL infected HFFs co-cultured with pDCs resulted in the production of twice these levels (800 pg/ml). Co-cultures of B virus or HSV-1 infected HFFs with pDCs, designed to reflect infected skin cells in vivo in the presence of pDCs in the vicinity, produced lower levels of IL-6 (100 pg/ml) when compared to the negative controls. The ability of pDCs to respond was verified by exposing pDCs to lysed infected cells, which resulted in production of 50 pg/ml of IL-6. Our co-cultures of B virus infected HFFs and pDCs induced twice as much IL-6 than pDCs directly exposed to virus in infected lysates, most likely because both pDCs and HFFs can respond to direct B virus exposure with the production of IL-6. Interestingly, HFFs treated with MCL produced 4500 pg/ml (M. Vasireddi, 2009), while MCL treated HFFs co-cultured with pDCs produced less IL-6 at 800 pg/ml, suggesting that HFF co-culture with pDCs may limiting the IL-6 production by HFFs or that nucleic acids released from lysed cell may be an important stimulant for activation of pDCs. Over-production of IL-6 can be detrimental to a host, so it is likely that pDCs or other cell types may regulate excessive production of IL-6. The suppression of IL-6 by B virus, relative to MCL, suggests either a direct or indirect virus-specific block or modulation of IL-6, which would benefit B virus survival and promote virus spread. The importance of
IL-6 in protection against zoonotic B virus infection is likely minimal as our positive control of HSV-1, an endemic human herpes virus that is rarely fatal, also suppressed IL-6 production when infected HFFs were co-cultured with pDCs. While the impact of IL-6 during B virus infection was not explored, pDCs can contribute to immune responses against B virus by activating skin cells, recruiting and activating macrophages, driving TH2 immune responses against B virus by promoting B cells differentiation. pDCs exposed to HSV-1 infected cell lysates produced IL-6 to a greater degree (5-fold) than pDCs exposure to B virus infected cell lysates. IL-6 has been shown by other investigators to be critical for resistance to HSV-1 in selected mouse strains [210]. Although the levels of IL-6 produced during virus infected co-cultures were similar between B virus and HSV-1, the role of IL-6 in zoonotic B virus infection remains to be investigated.

TNF-α (tumor necrosis factor or TNF), also produced by pDCs, is a highly inflammatory cytokine involved in the activation of many signaling pathways within both immune and non-immune cells. It is produced by many cell types including CD4+ T cells, NK cells, DCs, fibroblasts and endothelial cells and is a potent chemoattractant for neutrophils. TNF-α induces the production of cytokines and chemokines from many cells, stimulates phagocytosis in macrophages, activates apoptosis pathways, and is the key mediator of sepsis. TNF-α has been shown to be essential in preventing herpes simplex encephalitis in mice [152]. There are no data available on the production or impact of TNF-α during zoonotic B virus infection. As shown in the bottom panel of Figure 16, pDCs responded to the presence of B virus infected fibroblasts by producing significant amounts of TNF-α at 75 pg/ml (white bars). Our positive control of HSV-1 infected HFFs co-cultured with pDCs produced 187 pg/ml of TNF. Direct exposure of pDCs with B virus infected cell lysates, however, induced 406 pg/ml of TNF, indicating that pDCs were capable of producing greater levels when compared to those observed from co-cultures. The positive control of HSV-1 infected cell lysates induced 786 pg/ml, almost 2 times as much as pDCs directly exposed to B virus infected cell lysates. Our negative controls of medium (0.43 pg/ml) and MCL (0.58 pg/ml) did not
induce significant amounts of TNF-α in co-cultures of pDCs with HFFs, confirming that the response was specific to virus. In data not shown, B virus infection of HFFs alone failed to produce any TNF-α, confirming that production was from pDCs (M. Vasireddi, 2009). These results suggest that pDCs in the presence of zoonotically infected cells are a source of TNF-α, which would recruit and activate neutrophils, trigger phagocytosis of macrophages and induce apoptosis of infected cells. Future studies in more complex cell co-culture models are planned for the future, and these may lead to the development of an effective small animal model.
Figure 16 Cytokines produced from B virus-exposed pDCs

Human pDCs were isolated by negative selection from pooled PBMCs from several antibody-negative donors and exposed to B virus infected cell lysates or to B virus infected HFFs. pDCs produced significant levels (P<0.0001) of IL-1α, IFN-α, IL-6 and TNF-α in response to direct exposure to B virus infected lysates (gray bars), but pDCs produced significant levels (p<0.05) of only IFN-α and TNF-α in the presence of intact B virus infected HFFs (white bars). There was a significant (p<0.05) down-regulation of IL-6 from pDCs exposed to B virus infected HFFs when compared to MCL treated HFFs. Statistical analysis was performed on data from triplicate samples from one experiment using ANOVA with a post-hoc of Newman-Keuls **p<0.05 ***p<0.0001. Error bars represent mean of the standard error (SEM). The table shows the mean value of each cytokine and the ratio of the mean of pDC cytokine production when directly exposed to B virus lysates divided by the mean of pDC cytokine production in response to infected HFFs. ND=not detected, NA=not applicable.

Many cells produce chemotactic cytokines or chemokines as a form of communication, and for the recruitment of effector immune cells. pDCs are one of the first responders to invading viruses, so any chemokines produced by pDCs will help shape the immune response by directing the recruitment of effector cells. Therefore, we designed an experiment to measure chemokines produced by pDCs in re-
response to intact B virus infected HFFs or to direct exposure to B virus infected cell lysates. We tested the same supernatants that we obtained for the cytokine analysis described above and had the same negative controls of medium and MCL to verify the chemokines were specific to B virus, and HSV-1 was again used as a positive control. The data revealed for the first time that B virus infected HFFs fail to induce any significant amounts of the chemokines measured, except for IP-10. The pDCs were functional as direct exposure to B virus infected cell lysates induced significant amounts of IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4), and IP-10 (CXCL10). The lack of chemokine production by pDCs exposed to B virus infected HFFs provides a major roadblock, which can prevent the development of an efficient and effective immune responses, which is lacking in the fatal cases of zoonotic B virus infection. What is lost in the absence of each of these chemokines? To better understand, we summarize what each of these normally function to recruit.

IL-8 or CXCL8 is a chemokine produced by mononcytes, macrophages, fibroblasts, endothelial cells, dendritic cells and keratinocytes, and can be induced by IL-1 and TNF-α. IL-8 is a chemotactic protein that can recruit all migratory immune cells. It can activate neutrophils for the release of cytotoxic granules and can also influence the production of immunoglobulins from B cells. As shown in Figure 17, pDCs respond to B virus infected HFFs with 87 pg/ml of IL-8 production, and our positive control of HSV-1 infected HFFs produced 89 pg/ml, but the levels were significantly less than those measured in to the negative controls of medium (1500 pg/ml) and MCL (2500 pg/ml) co-cultures. In contrast, direct exposure of pDCs with B virus infected cell lysates produced 400 pg/ml of IL-8 and HSV-1 infected cell lysates induced 750 pg/ml of IL-8, which was nearly twice the levels produced by B virus exposed pDCs. These responses were specific to virus as both of the negative controls for direct exposure failed to induce any IL-8. The role of IL-8 in neutrophil activation is well known. Neutrophils play a major role in the early response to HSV-1 infection. While there are many cells capable of producing IL-8, it is important to note that pDCs contribute to neutrophil recruitment and activation. Previous work in our lab has shown that
B virus infected HFFs fail to recruit neutrophils in experiments with transwell co-cultures, whereas HSV-1 infected HFFs are able to recruit neutrophils under similar conditions. These results only tested the effect of one cell type, HFFs, so the data presented here suggest a more telling possibility of limited immunity, also observed in fatal zoonotic infections. The failure of pDCs to produce key recruiting chemokines such as IL-8 in co-cultures with infected HFFs is likely a critical step in developing immunity against this fatal virus and may be a target for therapeutic modulation.

MIP-1α or (CCL3) and the closely related MIP-1β (CCL4) are chemokines involved in the recruitment of immune cells such as T and B cells. They are produced by a number of cells, including macrophages, CD8+ T cells, B cells, neutrophils, dendritic cells, natural killer cell, keratinocytes, fibroblasts and many more. They are involved in recruitment of innate immune cells, in development of a TH1 response and in CD4+ T cell differentiation. They can also induce cytokine production from fibroblasts, specifically IL-1, TNF-α and IL-6. As shown in the second and third panels of Figure 17, pDCs in co-culture with B virus infected fibroblasts (white bars) failed to produce any significant levels of MIP-1α or MIP-1β at 0pg/ml and 4pg/ml, respectively. Similarly, neither negative control induced pDC production of significant amounts of MIP-1α or MIP-1β. Our positive control, pDCs co-cultured with HSV-1 infected fibroblasts also failed to induce MIP-1α, but induced 55pg/ml of MIP-1β. We know that the pDCs were functional because direct exposure (gray bar) with B virus infected cell lysates resulted in the production of 150 pg/ml of MIP-1α and 190 pg/ml of MIP-1β from pDCs, and our positive control, direct exposure of pDCs to HSV-1 infected cell lysates, resulted in the production of 600 pg/ml of MIP-1α and MIP-1β. These data indicate that pDCs in co-culture with B virus infected HFFs would not be able to recruit many different types of immune cells. Whether the levels of MIP-1α and MIP-1β produced from B virus exposed pDCs are sufficient for the recruitment of immune cells requires additional experiments.

IP-10 (CXCL10) also known as interferon gamma-inducible protein 10 is produced by a number of cells such as keratinocytes, lymphocytes, monocytes, dendritic cells and macrophages. Production of IP-
10 can be induced by IFN-γ or TNF-α and is a potent chemoattractant for monocytes, macrophages, T cells and DCs. Of the chemokines measured and as shown in the bottom panel of Figure 17, IP-10 was the only chemokine produced in significant levels (275 pg/ml) when pDCs were co-cultured with B virus infected HFFs. Our positive control, HSV-1 infected HFFs co-cultured with pDCs induced 300 pg/ml of IP-10, which was nearly equivalent to pDCs co-cultured with B virus infected HFFs. Our negative controls, pDCs co-cultured with HFFs exposed to medium or MCL also induced IP-10, but in significantly lower levels at 5 pg/ml and 91 pg/ml, respectively. HFFs exposed to MCL induced half as much of IP-10 than with MCL treated HFFs co-cultured with pDCs (data not shown; M. Vasireddi, 2009). pDCs directly exposed to B virus infected cell lysates produced robust amounts of IP-10 at 2500 pg/ml, 10 times as much as co-cultured pDCs, verifying that pDCs were functional. Our positive control, pDCs directly exposed to HSV-1 infected cell lysates produced 4000 pg/ml of IP-10, about 1.5 times more than pDCs directly exposed to B virus infected cell lysates. This response was specific to B virus as both of the negative controls of medium and MCL failed result in the production of induce IP-10 from directly exposed pDCs. The production of IP-10 was likely induced by TNF-α, as both B virus and HSV-1 exposed pDCs produced high amounts of TNF-α (406 pg/ml and 786 pg/ml, respectively), but failed to produce any detectable levels of IFN-γ. IP-10 is a potent recruiter of many innate immune cells and production by pDCs co-cultured with B virus infected HFFs would allow for recruitment and the development of an antiviral immune response against B virus, but whether these levels are sufficient requires further experiments.
Figure 17 Chemokines produced from B virus-exposed pDCs

Human pDCs were isolated by negative selection from pooled PBMCs from antibody negative donors and exposed to B virus infected cell lysates or to B virus infected HFFs. pDCs induced significant levels (P<0.0001) of IL-8, MIP-1α, MIP-1β and IP-10 in response to direct exposure to B virus infected lysates (gray bars), but pDCs only induced significant levels (p<0.05) of IP-10 in response to B virus infected HFFs (white bars). Statistics were triplicate of one experiment using ANOVA with a post-hoc of Newman-Keuls **p<0.05 ***p<0.0001. Error bars represent mean of the standard error (SEM). The table shows the mean of each chemokine and the ratio of the mean of pDC chemokine production when directly exposed to B virus lysates divided by the mean of pDC chemokine production in response to infected HFFs. ND=not detected, NA=not applicable.
3.2 HFFs in pDC co-cultures produce infectious B virus

In an effort to explain why pDCs co-cultured with B virus infected HFFs produced fewer and lower concentrations of the cytokines and chemokines than pDCs directly exposed to B virus infected cell lysates, we hypothesized that the amount of virus available to pDCs in the context of B virus infected HFF co-cultures was significantly less than that in direct exposure to B virus cell lysates. B virus virions are primarily cell-associated, as observed by the ability of B virus to form syncytia in multiple cell types, including Vero cells (Figure 5), HFFs, and rhesus macaque fibroblasts (RMFs). The fusion of infected cells with healthy cells is one virus strategy that helps to limit the numbers of free virions (not cell-associated) available for immune surveillance, but still allows virus to enter peripheral neurons, either by fusion of infected cells with neuronal membranes or escape of enveloped virions. To test this, we grew HFFs to subconfluency in a 12-well plate and infected them with B virus (MOI 5). Unadsorbed virus was removed after 1h and fresh culture medium was added and cultured these for an additional 23h. To quantify the amount of virus released from these infected cells we collected only the supernatants from infected HFFs after 24 h of infection (SUP). In addition, we collected both the HFFs and supernatants to quantify the amount of virus that is cell-associated and released into the supernatants (SUP+CELLS) from B virus infected HFFs after 24h of infection. We used a standard plaque assay to quantify the amount of virus within each sample, which is done by creating serial dilutions of the unknown samples, adding a known volume of each dilution onto confluent Vero cells, overlaying with methylcellulose and culturing for 48h. Plaques appear as spaces due to cell death and represent individual infectious virions, which after staining with crystal violet are counted and virus titers are determined. The results were calculated and expressed as the titers for 2x10⁴ HFFs, the same amount of HFFs that were infected in the co-culture studies. As shown in Figure 18, B virus titers in the supernatants only (HFF sup) were 1.8x10⁴ pfu, which was significantly less (p<0.05) by student t-test than the nearly 10-fold greater titer of HFF and supernatants (HFF sup+cells) at 1.3x10⁵ pfu. Based on these data, the amount of virus available to pDCs in co-cultures...
with infected HFFs was less nearly 4-fold less than that amount of B virus released from lysed infected cells for exposure to pDCs in direct exposure or $5 \times 10^4$ pfu (represented by the dashed line in Figure 18). This reduction in virus levels correlated with significantly reduced levels of cytokines and chemokines produced by pDCs in co-cultures with infected HFFs.

![B virus titers at 24hpi](image)

**Figure 18** B virus titers in human fibroblasts

HFFs were infected at an MOI of 10 in 12-well plates. After 24h, B virus titers in cell-free medium were measured by standard plaque assay to determine the amount of cell-free virus available to pDCs in the co-culture experiment described above. HFF sup resulted in $1.8 \times 10^5$ pfu and HFF sup+cells resulted in $1.3 \times 10^6$ pfu per $2 \times 10^4$ HFFs. The dashed line represents the titer of B virus that pDCs were exposed to B virus infected cell lysates ($5 \times 10^4$ pfu). HFF=human foreskin fibroblasts, sup=supernatant, sup+cells= supernatant and infected cells. Error bars represent SEM of triplicate samples. **p<0.05 using student’s t-test for comparison of the means values.

### 3.3 IFN-α from pDCs is dependent on B virus dose

To extend our experiments from looking at the amounts of B virus produced from HFFs during co-culture, we wanted to determine how different doses of B virus infected cell lysates would affect IFN-α production from pDCs. We chose IFN-α as the measure of the pDC response to B virus infected cell lysates because pDCs are the most potent producers of Type I IFNs in response to viral nucleic acids. Our previous experiment showed that the titers pDCs were exposed to in co-culture with B virus infected
HFFs were about 4 times lower than the titers of B virus released from infected cell lysates. Based on these data we hypothesized that **pDC-induced IFN-α correlated directly with virus dose to which these were exposed.** To determine the effect of virus dose on the ability of pDCs to produce IFN-α, freshly isolated pDCs from pooled PBMCs collected from seronegative donors were exposed to B virus at 2, 5, 10 and 20 pfu/cell. To ensure that the pDCs were responsive to B virus two positive controls were used, i.e., HSV-1 at 5 pfu/cell and CpG 2216 at 5μg/ml. The pDCs were seeded in 50ul of culture medium into 96-well round-bottomed plates and each treatment was added in 150ul of culture medium. pDCs were cultured at 37°C, in a 5% CO₂ humidified incubator, and at 24h cell-free supernatants were centrifuged at 100xg for 10min to remove cells and debris and tween/DOC was added to a final concentration of 1% to inactivate virus. Supernatants were stored at -80°C until analyzed by ELISA for IFN-α using the Human IFN-α pan ELISA Development Kit (HRP) (MabTech, Cincinnati, OH). This kit detects 11 of the 12 subtypes of IFN-α using a sandwich ELISA technique with a capture antibody and a detection antibody conjugated with horseradish peroxidase (HRP). Serial dilutions of a standard provided with the kit allowed for generation of a standard curve and unknown IFN-α concentrations were interpolated using RIA Spline/LOWESS in GraphPad Prism™. As shown in Figure 19, the pDCs responded to B virus released from the infected cells during the course of infection with the production of IFN-α although it was inversely correlated to the dose of B virus. Both of our positive controls verified that the pDCs were responsive by producing IFN-α in response to HSV-1 infected cell lysates and CpG 2216 with 33 fg/cell and 42 fg/cell, respectively. The pDCs exposed to B virus released from infected cell lysates at 2 pfu/cell (or 2x10⁵ pfu) produced 33.5 fg/cell of IFN-α, while pDCs exposed to B virus released from infected cell lysates at 5 pfu/cell (6x10⁶ pfu) produced 28 fg/cell of IFN-α, while high MOIs of B virus released from infected cell lysates at 10 pfu/cell (1.2x10⁵ pfu) and 20 pfu/cell (2.4x10⁵ pfu) failed to induce significant amounts of IFN-α at 4 and 5 fg/cell, respectively. Neither of the two negative controls, cells exposed to medium or MCL (at the protein equivalent to BV 20 pfu/cell), produced any IFN-α, indicating that the
IFN-α produced was a specific response to virus. This result was unexpected because independent studies with HSV-1 have shown that the more virus, the greater the amount of IFN-α pDCs produce, indicating that high MOIs of B virus may block or overwhelm pDC IFN-α production. These data support our hypothesis that IFN-α production by B virus exposed pDCs correlates inversely with the amount of virus used to expose pDCs, surprisingly. Based on these data and the data shown in Figure 18, the pDCs in co-cultures with B virus infected HFFs (1.8x10⁴ pfu) should produce similar amounts of IFN-α as pDCs in direct exposure to B virus infected cell lysates (5x10⁴ pfu) as there was no significant difference of IFN-α produced from pDCs exposed to 2 or 5 pfu of B virus, both of which had significantly higher production of IFN-α than pDCs exposed to MCL (Figure 19). To explain the drastic reduction of IFN-α production from pDCs in co-cultures when compared to pDCs directly exposed to B virus infected cell lysates, we considered the quality of virus within the virus lysates as these virions are likely surrounded by the nuclear or Golgi membranes whereas the virions released from infected cells in co-cultures would be surrounded by plasma membranes acquired when virions egress out of infected cells by exocytosis. Another possible explanation for the increase of IFN-α in pDCs directly exposed to B virus infected cell lysates is that these contain free viral DNA that is available for the pDCs to endocytose and activate TLR9 dependent IFN-α production. There is likely to be very little free DNA in the co-culture system, as only intact virions would be released with the DNA protected within the enveloped capsid. Future studies are needed to fully understand the mechanism of a differential response between B virus infected cell lysates and the B virus virions produced from infected HFFs in co-cultures.
**Figure 19 IFN-α production from pDCs is inversely correlated to virus within infected cell lysates**

pDCs were isolated by negative selection from pooled, fresh PBMCs obtained from seronegative donors and were distributed equally into wells of a 96-well plate. pDCs were exposed to B virus in infected cell lysates with titers ranging from 2-20 pfu/ml, as determined by standard plaque assay. pDCs were cultured for 24h and cell-free supernatants were collected and tween/DOC, added to a final concentration of 1%. IFN-α levels were determined using ELISA. As negative controls, medium treated pDCs and MCL treated pDCs, at a level equivalent to BV 20 pfu, were used. As positive controls, pDCs were exposed to HSV-1 at 5 pfu/cell and pDCs were exposed to 10µg/ml of CpG 2216. **p<0.05, NS=not significant.

ANOVA was used to analyze results along with Newman-Keuls post hoc test for pair comparisons.

3.4 pDCs do not support productive B virus replication

We showed that pDCs exposed to high titers (>10 pfu/ml) of B virus fail to produce significant amounts of IFN-α (Figure 19). The viability of pDCs after B virus exposure was unchanged for all titers (data not shown, nor were any cytopathic effects (CPE) apparent by microscopic analysis. Based on the reduction in IFN-α production from pDCs after exposure to high titers of B virus from lysed infected cells, we hypothesized that B virus infects pDCs, perhaps abortively since no CPE was observed. To test this hypothesis, we exposed purified pDCs to B virus infected cell lysates (MOI 10) in 50ul in medium and cultured for 1h at 37°C, 5% CO₂ to allow for adsorption. After 1h, 150ul of medium was added and pDCs were cultured for an additional 23h. To accurately quantify any changes in B virus titers after 24h, input virus used to infected pDCs along with harvested supernatants were stored at -80°C for analysis. Samples were freeze-thawed two times prior to titering by standard plaque assay on Vero cells. As shown in Figure 20 Box A, the titer of B virus in pDCs after 24h was 2x10⁵ pfu/ml while the input titer was 7x10⁵.
pfu/ml at 1hpi. The reduction of virus after 24h was not significant by paired student t-test, but clearly shows that pDCs do not support productive infection. This slight reduction in titer was expected because dendritic cell can endocytose and destroy virus particles.

Prior to entry, B virus attaches to the surface of the cell via specific receptors. This stage of virus infection is referred to as adsorption in which virus particles are bound to receptors but have not entered the cells. To determine if the titers from pDCs at 24h were due to replicating virus, or because virus particles were bound to the surface and had not entered the pDCs, we modified the experiment to include a 50mM citric acid wash, which inactivates any bound virus particles that have not entered the pDCs. The same experimental setup was used except with the addition of a citric acid wash for 10min after the 1h adsorption step. pDCs were washed twice prior to re-suspension in 200ul of medium and incubated at 37°C for 23h and titers were quantified by standard plaque assay. As previously, an input titer was collected at 0hpi, upon the addition of B virus to the pDCs. As shown in Figure 20 B, the titers in pDCs after 24h was reduced to zero and the input titers were 2x10^5 pfu/ml. These data indicate that B virus failed to replicate within pDCs and the titers previously observed at 24hpi, were likely adsorbed virus particles that failed to enter cells, remaining bound to the pDC surface.

To further investigate whether pDCs support early events of virus infection and subsequent viral DNA replication, we quantified B virus replication by measuring B virus DNA copy numbers. To do this, we exposed pDCs to B virus (MOI 10) for 1h at 37°C, in a humidified 5% CO₂ incubator. After adsorption, unattached virus was removed by centrifugation at 400xg for 10 min. pDCs were re-suspended in 200ul of medium and incubated for an additional 23h, after which and cells and supernatants were collected in an equal volume of Solution A provided in the Invitrogen DNA Isolation Kit, and DNA was isolated according to the manufacturer instructions. The gene encoding glycoprotein B (gB) was amplified as a marker of DNA replication as the greater the numbers of viral genomes replicated, the more copies of gB DNA. Quantitative real-time PCR was used to assess the numbers of gB DNA copies present within B
virus exposed pDCs, both in our input collected at 1hpi and output 24hpi. The gB gene was amplified using forward and reverse primers and detected using the internal sequence specific probe of FAM-TAMRA. To quantify the gB copy numbers in pDCs exposed to B virus, a standard curve was generated using known concentrations of plasmids containing the gB gene. As shown in Figure 20C, the number of gB DNA copies in pDCs at 24h was $3.5 \times 10^8$ and in pDCs at 1h was $2 \times 10^8$. The increase in DNA copy numbers at 24h was not significant by paired student t-test further confirming that B virus does not productively infected pDCs.

In all of the experiments so far we have measured late stages of viral infection, either intact virion production (plaque assay) or DNA replication (gB DNA). Presumably in the absence of viral DNA replication, early events post-infection have been blocked to some extent. To determine whether B virus abortively infects pDCs, we used a standard reverse-transcriptase PCR to measure viral gene expression at throughout infection. To do this, we exposed pDCs to B virus (MOI 2) for 2h, 4h, or 6h at 37°C, in a humidified 5% CO$_2$ incubator. Because we were amplifying RNA transcripts, it was not necessary to remove virus after adsorption. pDCs were incubated for the respective times and cells were pelleted by centrifugation at 400xg for 10min. The cell pellets were resuspended in 200ul Tri-Reagent and RNA was isolated according to the manufacturers instructions. A One-Step reverse transcriptase PCR was conducted to convert RNA into cDNA, then using specific primers for B virus genes of immediate-early (ICP0), early (TK) and late (gC) were amplified. Amplification samples were electrophoresed on a 1.5% acrylamide gel containing ethidium bromide for DNA detection. As a positive control, we used HFFs infected for 24h with B virus (MOI 10) to confirm transcript detection. A negative control of water was used to detect contamination. ICP0 is an immediate-early (IE) gene and during productive infection the expression reaches peak levels at 2-4hpi. As shown in Figure 20D, ICP0 gene expression in B virus exposed pDCs was restricted, as a very light band was visible at 4 and 6hpi, but was not near the intensity of our positive control. Thymidine kinase (TK) was used as a measure of early gene expression, which
during productive infection peaks around 6hpi. At no time point in B virus exposed pDCs was TK gene expression detected, but it was present in the positive control. Lastly, gC was used as measure of late gene expression. We expected to see no gC transcripts since we did not detect any viral replication (gB DNA) or infectious virions (plaque assays) and as expected we did not see gC transcripts in B virus exposed pDCs. These data indicate that pDCs do not support productive replication of B virus and infection is abortive at the IE stage of the B virus life cycle. Combined, all of these data confirm that B virus does not productively infect pDCs, since B virus titers are not increased after 24h (Figure 20A and B), that B virus gB copy number do not increase significantly after 24h (Figure 20C), and that early and late B virus genes are not expressed (Figure 20D). Based on these data, we can conclude that the lack of IFN-α production from pDCs exposed to high titers of B virus was not due to virus replication.
Figure 20 pDCs do not support productive B virus infection
A. pDCs were cultured with B virus (MOI 10) for 0 and 24h and B virus titers were measured by standard plaque assay. B virus titers were not reduced significantly within 24hpi.
B. pDCs were cultured with B virus (MOI 10) for 1h, then washed with citric acid and cultured for 23h and B virus titers were measured by standard plaque assay. B virus titers were reduced to zero after 24h.
C. pDCs were cultured with B virus (MOI 10) for 1h, unadsorbed virus was removed and cultured for 24h. DNA was purified and qRT-PCR was used to measure gB DNA copy numbers, which were not significantly increased after 24h.
D. pDCs were cultured with B virus (MOI 2) for 2, 4, and 6h. RNA was isolated from cell pellets. B virus transcript expression was detected using reverse-transcriptase PCR and run on an acrylamide gel. A positive control of B virus infected HFFs were used. NS=not significant.

3.5 Macaque and human PBMCs fail to produce IFN-α in response to B virus infected fibroblasts

Understanding the role of individual immune cell types is important for defining direct effects of virus infection, but there are many indirect effects mediated by the interplay with and between other defense cells. The pDCs are a specific subset of dendritic cells that as we have shown in Figures 16 and 17, respond to B virus infected fibroblasts by producing IFN-α, TNF-α and IP-10. We have reviewed how each of these molecules impacts other defense cells. The levels of each is limited, however, when compared to direct exposure to B virus infected lysates after which human pDCs produced significantly more
IFN-α, TNF-α, and IP-10, as well as IL-1α, IL-6, IL-8, and MIP-1α/β. The restriction of cytokine production in human pDCs exposed to infected cells was not due to limited virus exposure due to syncytial formation in fibroblasts, because we have shown that titers were similar in the supernatants of co-cultures and infected cell lysates (Figure 18) or due to B virus infection of pDCs (Figure 20), which we have shown is non-productive. One of our original purposes of the research presented in this dissertation was to compare the natural and foreign host response to B virus in relationship to role(s) of pDCs. Macaques are the natural host for B virus and the data available on how they respond to B virus is extremely limited. To contribute to the overall knowledge of the macaque response to B virus, we explored the macaque pDC response to B virus, however, pDC studies in macaques are difficult due to limited availability of blood and a lack of pDC isolation reagents and experimental infections require BSL-4 facilities. To measure the pDC response to B virus in macaques using our cell culture model, we measured IFN-α production from macaque PBMCs exposed to B virus infected cell lysates and compared these data to the IFN-α production from pooled human PBMCs (Figure 21) and human pDCs (Figure 19) exposed to B virus similarly. Because there are no available reagents for the isolation of macaque pDCs, and because macaque PBMCs contain similar numbers of pDCs as humans [211], we used IFN-α production from human PBMCs (Figure 21) to correlate the amounts of IFN-α produced from pDCs (Figure 19) in macaque PBMCs. Human pDCs produced about 30 fg/cell of IFN-α when exposed to an MOI of 5 (Figure 19), and assuming that pDCs are 0.1% of total PBMCs, the PBMCs made approximately 46 pg/ml (46,000 fg/ml; or 46 fg/pDC; Figure 21), which was nearly equivalent to the IFN-α produced by purified pDCs. These data support numerous observations that pDCs produce the majority of IFN-α in spite of the fact that they comprise less than 0.1-0.8% of the PBMCs. Based on these data, IFN-α production from macaque PBMCs was used to correlate pDC cytokine production in macaques. The additional IFN-α produced from B virus exposed human PBMCs can be attributed to other cell types that make IFN-α such as myeloid dendritic cells, but we did not explore these specific sources of IFN-α in our studies. The percentage of immune
cell types in PBMCs varies depending on individual immune responses. Whole blood contains leukocytes (immune cells), erythrocytes (red blood cells, RBC) and platelets. Ficoll-Density Centrifugation allows the separation of leukocytes from other blood products. Leukocytes comprise approximately 0.1-0.2% of blood cells. Of these leukocytes, T cells make up approximately 7-14%, B cells make up 1-7%, NK cells are 1-6%, myeloid cells, which include DCs, make up 53-86% and monocytes make up 2-12%. The granulocytes (35-80%) are typically removed from the leukocyte population during gradient centrifugation as they pellet with RBCs and platelets. We measured IFN-α production from macaque PBMCs following direct exposure to B virus infected cell lysates and in co-cultures with B virus infected RMFs. Based on the characteristic of the rapid and robust induction of neutralizing antibodies in macaques after B virus infection, we hypothesized that B virus infected macaque co-cultures would induce robust amounts of IFN-α from PBMCs, while human co-cultures would be restricted.

Rhesus macaque fibroblasts were not commercially available at the time of these studies, so in order to test the macaque response we first had to establish and characterize a rhesus macaque fibroblast cell line from whole skin biopsies obtained from Yerkes Primate Center. We designate these are rhesus macaque fibroblasts or RMFs. The methods for isolation of RMFs will not be discussed here but are detailed in the materials and methods. Immunofluorescence was used to confirm fibroblast purity using the fibroblasts markers vimentin and fibronectin, as well as cytokeratin, a keratinocyte marker (Figure 31 in Appendix). PBMCs were isolated under sterile conditions from fresh macaque or human blood using Ficoll-Hypaque gradient centrifugation and stored in liquid nitrogen. At the time of the experiment, PBMCs were quickly thawed, checked for viability and used immediately. For each experiment, PBMCs were used from one individual macaque or human.

To test our hypothesis we setup a co-culture system similar to what we described previously for human pDCs (Figure 15). RMFs and HFFs were seeded into 96-well plates, grown to sub-confluency and infected with B virus (MOI 5). At 1hpi, unadsorbed virus was removed and PBMCs were added to each
well in 200 ul medium and incubated for 23h. Simultaneously, PBMCs were seeded in 50ul of culture medium into 96-well round-bottomed plates and each treatment was added in 150ul of culture medium. Cells were incubated at 37°C, in a humidified 5% CO₂ incubator and at 24hpi cell-free supernatants were collected by centrifugation at 400xg for 10min. Tween/DOC was added to a final concentration of 1% to inactivate virus. Supernatants were stored at -80°C until analyzed by ELISA to measure IFN-α levels using the Human IFN-α pan ELISA Development Kit (HRP) (MabTech, Cincinnati, OH). This kit detects 11 of the 12 subtypes of IFN-α using a sandwich ELISA technique with a capture and a detection antibody conjugated with horseradish peroxidase (HRP). Serial dilutions of a standard provided with the kit allowed for generation of a standard curve and IFN-α concentrations were interpolated using RIA Spline/LOWESS in GraphPad Prism™. To ensure the functionality of the PBMCs, we used positive controls known to induce IFN-α from PBMCs. For human cells we used HSV-1, as this virus is endemic in humans as B virus is endemic in macaques, and it is a closely related simplex virus, and a robust inducer of IFN-α from PBMCs. For macaque cells, CpG 2216 (5 μg/ml), which is a synthetic DNA molecule that activates pDCs via TLR9, was added at 1hpi to co-cultures. CpG was used primarily because HSV-1 does not infect RMFs productively. As negative controls, medium only and MCL were used to measure any basal response(s) or to how the virus stocks were generated.

As shown in Figure 21 Box A, B virus infected RMFs co-cultured with PBMCs (white bars) failed to produce significant amounts of IFN-α at 11.69 pg/ml. Our positive control of CpG 2216 verified that macaque PBMCs in co-cultures were capable of producing IFN-α with mean levels of 65 pg/ml. When macaque PBMCs were directly exposed (gray bars) to B virus infected lysates or CpG, each induced 24 pg/ml proving that the PBMCs were responsive to B virus. As expected, neither of the negative controls induced significant levels of IFN-α in co-cultures with RMFs or from direct exposure.

Similarly and as shown in Figure 21 Box B, human PBMCs co-cultured with B virus infected HFFs failed to produce any significant amount of IFN-α at 1.79 pg/ml, but our positive control of HSV-1 shows
that PBMCs were responsive by producing 6.48 pg/ml of IFN-α. Also, human PBMCs directly exposed to B virus or HSV-1 infected lysates produced 46.59 pg/ml and 107 pg/ml of IFN-α, respectively. The response was virus specific as human PBMCs that were directly exposed or in co-cultures with negative controls did not produce significant amounts of IFN-α. It is important to point out that neither human or macaque fibroblasts produced any detectable IFN-α in response to any of the treatments (data not shown, Brock 2012).

These data show that the host specific PBMC response to B virus infected fibroblasts was similar between human and macaques. There was a difference in the amount of IFN-α produced from PBMCs after direct exposure to B virus infected lysates. Human PBMCs made twice as much IFN-α than macaque PBMCs after direct exposure. Macaques have similar numbers of pDCs in PBMCs as humans [211], but contain fewer mDCs [212], which may explain the observed reduction in the amount of total IFN-α from macaque PBMCs directly exposed to B virus. Using our correlation of human PBMC induced IFN-α with that of pDCs, we can assume because macaques contain a similar number of pDCs at 0.1% of PBMCs, that the IFN-α should be equivalent to human pDCs. The level of IFN-α produced from macaque PBMCs, however, was half as much as from human PBMCs, indicating that macaque pDCs may respond differently to B virus, or alternatively, because they have fewer mDCs, show an overall reduction in IFN-α.

There are limited data available on the PRRs in macaque cells as compared to human cells, but macaque pDCs express similar levels of TLR7/9 and IRF7 and respond to HSV-1 by producing similar levels of IFN-α, when measured by intracellular cytokine staining and ELISPOT [211]. These data suggest that macaque cells are sensing B virus or viral DNA differently than human pDCs/PBMCs. If this is the case, then it is crucial to understand the differences between these two hosts, especially considering that macaques are critical animal models for many human diseases, and with respect to this dissertation, could explain the different outcomes of infection, providing a potential target for therapeutics.
To determine whether the amount of virus available in the supernatants of infected fibroblasts affected the IFN-α production by PBMCs, we compared the amounts of B virus available to PBMCs in co-cultures. To test this, HFFs and RMFs were infected with B virus (MOI 5) and B virus titers were measured in the supernatants after 24h. B virus remains primarily cell-associated through the fusion of infected cells (syncytia), which limits the amount of cell-free virus within supernatants. As shown in Figure 22, the titers in supernatants from B virus infected RMFs was $4.6 \times 10^4$ pfu/ml, while the titers of B virus in supernatants of infected HFFs was $1.8 \times 10^4$ pfu/ml, a 2.5-fold difference. B virus titers were much higher in RMFs within the cell and supernatants than HFFs, as expected. The titer of B virus in infected cell lysate-exposed exposed PBMCs was $5 \times 10^5$ pfu/ml, approximately a log greater than in the co-cultures. The titers of B virus in the supernatants from RMFs and HFFs were similar, and although the levels of IFN-α produced in co-cultures were not significant, RMFs produced nearly 12 pg/ml while human cells produced only 2 pg/ml. This 3-fold reduction in virus production from HFFs may explain, in part, the reduction in the levels of IFN-α produced from human PBMCs in co-cultures. A dose response study is required to quantify the ability of PBMCs to make IFN-α in co-cultures. As discussed previously, the quality of virus produced from infected fibroblasts may be different than the virus accessible in the B virus infected lysates used in direct virus exposure. Additionally, and perhaps more importantly, free-viral DNA is likely present in the infected cell lysates, but this was not measured in our experiments to date.
**Figure 21 IFN-α production from B virus-exposed human and macaque PBMCs**

HFFs (human) and RMFs (macaques) were infected with B virus (MOI 5) for 1h and PBMCs were added and cultured for 24h (White bars). Simultaneously, human and macaque PBMCs were directly exposed to B virus infected cell lysates (MOI 5), as well as to control treatments and cultured for 24h (Gray bars). Cell-free supernatants were collected, tween/DOC was added to a concentration of 1% to inactivate viruses and IFN-α concentrations were measured by ELISA. UN=medium only; MCL=uninfected cell lysate; HSV-1 MOI of 5; CpG 2216 (5μg/ml). Error bars represent SEM. *p<0.05 by ANOVA with a post-hoc of Newman-Keuls.
Infectious B virus contained in supernatants/cells of B virus infected human and macaque fibroblasts

B virus titers were measured in the supernatants (sup) and in the supernatants and cells (sup+cells) after 24 hpi to measure progeny virus from B virus infected (MOI 10) rhesus macaque fibroblasts (RMFs), and human foreskin fibroblasts (HFFs). The supernatants from HFFs contained $1.8 \times 10^4$ pfu/ml of B virus while the supernatants from RMFs contained $4.6 \times 10^4$ pfu/ml of B virus. The virus is primarily cell-associated as shown by the titers obtained from supernatants together with cells at $1.3 \times 10^5$ pfu/ml for HFFs and $1.6 \times 10^6$ pfu/ml for RMFs.

3.6 B virus replication is not sensitive to IFN-α produced from virus exposed PBMCs

We have here that human pDCs, as well as human and macaque PBMCs respond to direct B virus exposure with the production of cytokines and chemokines, albeit the levels of these are lower than those observed in parallel experiments with HSV-1. We have further evaluated in greater detail, IFN-α production by pDCs exposed to infected fibroblasts versus pDCs exposed to B virus released from infected cell lysates. The levels of cytokines produced by human pDCs, or human and macaque PBMCs, co-cultured with B virus infected fibroblasts were significantly lower than the levels in parallel experiments infected with HSV-1 (Figures 16 and 21). These data demonstrate that pDCs are activated by B virus more so in the presence of infected cell lysates, than in the presence of intact infected cells, however, pDCs are not activated to the extent that they are in the presence of HSV-1 under similar conditions. To explore further why B virus infected cell lysates activated pDCs to a greater extent than intact infected...
cells, we speculated that perhaps there was less virus released by the intact infected cells. An explanation for the differences in pDC activation is not evident, but we have shown it is not due to limited amounts of virus in supernatants of infected fibroblasts (Figures 18 and 22). IFN-α is the classic antiviral compound and has been shown to dramatically reduce HSV-1 titers in vitro. Animals or humans with defects in their ability to produce IFN-α develop encephalitis as a result of HSV-1 infection, which is normally an innocuous infection in humans. To determine whether there was an antiviral effect of the IFN-α produced from human and macaque PBMCs exposed to B virus released from infected cell lysates, we quantified the sensitivity of B virus replication to PBMC produced IFN-α, with the knowledge that our data support that the bulk of IFN-α was produced from pDCs contained within the PBMC population. Supernatants from B virus exposed PBMCs either directly exposed or in co-culture with B virus infected fibroblasts, were tested to determine whether B virus titers from infected fibroblasts were reduced. We hypothesized that the IFN-α produced from B virus-exposed PBMCs will reduce the production of infectious virus from infected fibroblasts, but because PBMC co-cultures lack significant levels of IFN-α these will fail to reduce production of infectious B virus. To test this hypothesis, PBMCs were incubated with B virus contained within infected cell lysates (MOI 5) for 1 h in a minimal volume of culture medium supplemented with 2% FBS, after which the cell-culture medium volume was brought to 200 ul and incubated. After 24 h, cell-free supernatants were collected by centrifugation at 400xg for 10 min, in which any adsorbed virus particles would remain with the cells. RMF and HFFs were grown to sub-confluency in a 48-well plate and infected with B virus (MOI 5). After 1 h adsorption, the inoculum was removed and the supernatants previously collected from the B virus exposed PBMCs were added and cells were incubated for 23 h. These supernatants contained whatever IFN-α was produced by the PBMCs in response to exposure to B virus infected cell lysates. Infected RMFs and HFFs as well as supernatants from these were collected, frozen and thawed once, and titered by standard plaque assay. As a control, fibroblasts were treated similarly and cultured without PBMC supernatants. As shown in
Figure 23, neither human or macaque B virus exposed PBMC supernatants caused a significant reduction in the levels of infectious virus produced from each respective cell line even in the presence of IFN-α produced by PBMCs directly exposed to B virus (Figure 21). As shown in Figure 23A, B virus titers from human fibroblasts (A. left graph) at 24 hpi, following culture with supernatants harvested from B virus exposed PBMC (BV-exposed PBMC) were 5.4x10^5 pfu/ml, while HFFs treated with MCL-exposed PBMC (MCL-exposed PBMCs) produced 9x10^5 pfu/ml, a 0.6 times increase of titer, which was not significant when analyzed by student t-test. Macaque fibroblasts (A. right graph) showed a similar trend, but the titers were a log higher than HFFs for when the medium from MCL-exposed PBMC was cultured with infected RMFs (MCL-exposed PBMC) at 1.2x10^7 pfu/ml and when B virus-exposed PBMCs medium was incubated with infected RMFs (B virus-exposed PBMC) there was 1.5 times reduction in titers to 8x10^6 pfu/ml. B virus titers in both human or macaque fibroblasts when treated with medium from B virus exposed PBMCs were not significant when compared to infected fibroblasts that were treated with medium from MCL treated PBMC when analyzed using a paired two-tailed Student t-test. These data show that PBMCs exposed to B virus did not significantly inhibit B virus replication in spite of IFN-α production by B virus exposed PBMCs (Figure 21).

A more accurate representative of natural infection is to determine if supernatants from co-cultures of PBMCs could suppress B virus replication. We did not expect B virus replication to be reduced when infected fibroblasts were treated with the supernatants from B virus infected co-cultures, as they did not produce significant amounts of IFN-α (Figure 21). To test this, we infected fibroblasts with B virus (MOI 5) and at 1 hpi, we added PBMCs and cultured for 23 h. As a negative control, we MCL treated co-cultures at the equivalent and same way as B virus. Cell-free supernatants were collected by centrifugation at 400xg for 10 min. HFFs and RMFs were grown to confluency in a 48-well plate and infected with B virus (MOI 5). At 1hpi, unadsorbed virus was removed and the PBMC co-culture supernatants were added to infected fibroblasts and cultured for 24 h. As shown in Figure 23B and as expected, B vi-
rus titers were not significantly reduced when cultured with supernatants from B virus infected PBMC co-cultures. B virus titers in HFFs (B. Left graph) treated with supernatants from PBMCs co-cultured with B virus infected HFFs (BV PBMC Co-cultures) and MCL infected HFFs (MCL PBMC Co-cultures) showed a similar titer at $3.0 \times 10^5$ pfu/ml. Again, the trend was conserved in macaques as the titers for RMFs treated with supernatants from B virus infected co-cultures (BV PBMC Co-cultures) or MCL treated co-cultures (MCL PBMC Co-cultures) were the same at $3.0 \times 10^6$ pfu/ml, a log higher than HFFs. These data were what we expected because the co-cultures did not produce significant amounts of IFN-α. Overall, these data indicate that B virus exposed PBMCs, either directly exposed to B virus, or in co-cultures with infected fibroblasts, fail to limit B virus replication in fibroblasts in either host. These data were unexpected especially for macaque PBMCs because B virus is endemic in these animals, we predicted that these could limit B virus replication in cells derived from the primary sites of infection. These data make it clear that B virus is resistant to PBMC induced IFN-α in either host.
Figure 23 B virus exposed PBMC supernatants do not reduce B virus titers in fibroblasts

A. Supernatants from B virus exposed PBMCs do not reduce replication in B virus infected fibroblasts. PBMCs were exposed to B virus (MOI 5) or MCL for 24h. The supernatants were transferred to B virus infected fibroblasts (MOI 5) at 1hpi and cultured for 23h and titers were measured (BV-exposed PBMC). A negative control of MCL treated PBMCs were used (MCL-exposed PBMC). Titers are shown in the table on the right.

B. Supernatants from B virus infected PBMC co-cultures do not reduce B virus replication in infected fibroblasts. Fibroblasts were infected with B virus (MOI 5) and at 1hpi, PBMCs were added and cultured for 23h. Cell-free supernatants were transferred to freshly infected fibroblasts (MOI 5) and cultured for 23h and titers were determined. The medium from co-cultures of PBMCs with B virus infected fibroblasts was transferred to B virus infected fibroblasts (BV PBMC Co-cultures) and titers were measured after 24h. As a control, B virus infected fibroblasts were treated with MCL treated fibroblasts co cultured with PBMC (MCL PBMC Co-cultures). The tables on the right show the titers at each treatment.

3.7 Sensitivity of B virus replication in infected macaque and human cells to recombinant IFN-α

To consider the possibility that B virus replication may not be sensitive to the levels of IFN-α produced by PBMCs exposed to B virus infected cell lysates (46 pg/ml), we considered, with the high MOI of 5, that the IFN pathway, overwhelmed by large amounts of virus, could not effectively block the production of B virus progeny in newly infected cells. We hypothesized that the efficacy of the IFN-α antiviral activity is dependent on the IFN-α dose and the amount of infectious virus to which a cell is exposed.

To test this hypothesis, we pre-treated fibroblasts with universal Recombinant Type I IFN (PBL Interferon Source, Piscaway, PA) at varying concentrations ranging from 12.5-50 U/ml for 30 min prior to infection. The IFN-α treated HFFs and RMFs were then infected with B virus at a low MOI of 0.01 or high MOI of 5.
Unadsorbed virus was removed and cells were cultured in the presence of IFN-α for an additional 23 h. Cells were collected by scraping into the medium followed by freeze-thawing and titering by standard plaque assay. The purpose of using a low MOI was to allow for a natural scenario where IFN-α binds to bystander cells to provide some protection by blocking virus replication, whereas the high MOI was used to ensure maximal infection of all cells to determine whether IFN-α could block de novo production of progeny virus.

As shown in Figure 24, B virus replication in HFFs or RMFs was not significantly reduced, whether the infection was a high or low MOI, and regardless of the dose of IFN. B virus titers in HFFs infected at an MOI of 5, in the absence of IFN-α treatment were 2x10^6 pfu/ml. At the lower MOI of 0.01, HFF titers were reduced to 2x10^5 pfu/ml without IFN-α treatment. Virus titers were reduced to 5x10^4 pfu/ml, a 4-fold reduction, when HFFs infected (MOI 0.01) were treated with 12.5 U of IFN-α, but this reduction was not significant when analyzed by ANOVA. At 25 U and 50 U, the virus titers in HFFs at an MOI of 0.01 were nearly the same as untreated at 1x10^5 pfu/ml and 3x10^5 pfu/ml, respectively. At higher MOI 5 infections, infectious virus produced in HFFs, that were not treated with recombinant IFN-α, were present at a log higher than at the lower MOI of 0.01, with a similar drop in titers in the 12.5 U IFN treated group at 8x10^5 pfu/ml, but was not significant when analyzed using ANOVA. RMF titers at MOI 0.01 and MOI 5, were nearly the same without IFN-α treatment and at all doses of recombinant IFN-α, but were not significantly different at 1x10^7 pfu/ml. We expected the amount of virus progeny to be reduced in response treatment of cells prior to and throughout infection with higher doses of recombinant IFN-α particularly when infections were performed with low MOIs but the response was similar regardless of the amounts of recombinant IFN-α and virus in both host cell species. The levels of recombinant IFN-α in each of the treatments were much higher than we observed from our PBMCs (Figure 21). According to the manufacturer, PBL Interferon Source, 1 U of Universal Type I IFN has an activity of approximately 38 pg/ml of IFN, so 12.5 U represents 475 pg/ml of IFN, which we did not observe in any of the PBMC
cultures, either directly or in co-cultures, however it is shown later that these low levels are sufficient to activate STAT-1, which is downstream of the IFNαR. Even at artificially high levels of recombinant IFN-α, B virus replication was not significantly reduced in either macaque or human cells. We expected that B virus titers would be lower in the natural host cells, but these data clearly show that IFN-α does not block B virus replication. This is an important observation suggesting that the amount of virus produced during infection is likely a factor in different pathogenesis outcomes in macaques and humans, but rather other controllers or lack thereof, of the effects of produced virus play more important roles in the differential pathogenesis observed between macaques and humans in vivo, because at least at the site of entry, virus replication is unfettered in the presence of PBMC produced IFN-α or recombinant IFN-α.

**Figure 24** B virus titers are unaffected by recombinant IFN-α.
Fibroblasts were pretreated with Universal Type I IFN for 30min, after which they were infected with B virus at different MOIs. Cells were cultured for 24h with IFN and titered by standard plaque assay to quantify progeny virus produced from macaque and human fibroblasts. NS=not significant by student t-test.
3.8 Medium from B virus exposed PBMCs activates the Type I IFN signaling pathway in uninfected macaque and human fibroblasts

We have shown that B virus titers were not reduced in HFFs or RMFs when cultured with medium from B virus exposed PBMCs (Figure 23), so we wanted to know next whether IFN-α produced by B virus exposed PBMCs (Figure 21) is functionally active and sufficient to activate the IFN-α signaling pathway in uninfected fibroblasts. Because the titers of B virus were unaffected in the presence of IFN-α in B virus exposed medium from PBMC exposed to B virus infected cell lysates, we hypothesized that B virus induced IFN-α produced by PBMCs is not functional and does not activate the IFN-α signaling pathway in healthy fibroblasts from either macaque or human species. Under normal, healthy conditions, type I IFN signaling pathway is activated upon binding of IFN-α/β to the IFNαR. HSV-1 has been shown to target the IFN receptor for degradation as a mechanism to limit the IFN response [213], but when HFFs and RMFs were infected with B virus (MOI 5), the IFNaR levels were not changed when compared to MCL treated cells (data not shown, N. Brock, 2012). Ligand:receptor binding activates Janus kinases (JAK), of which there are four members (JAK1, JAK2, JAK3, and TYK) to recruit and phosphorylate STATs, of which there are 7 family members (STAT1-6; STAT 5a, 5b). Activated STATs then form a homo- or heterodimer that complex with an interferon regulatory factor to become an interferon stimulated gene factor (ISGF). In the case of Type I IFN signaling, the transcription factor complex is designated ISGF3, which is composed of a heterodimer of STAT1/2 and IRF9. The ISGF enters the nucleus via specific nuclear membrane transporters and binds to specific promoter regions within DNA designated interferon response elements (ISRE). The trans-activating domain, located at the C terminus of the STAT molecules can recruit other transcription factors and co-factors to mediate interferon-dependent gene expression. Once the genes are activated, phosphatases within the nucleus can remove the phosphate groups from the STATs to turn off gene transcription. The de-phosphorylated STATs are shuttled back to
the cytoplasm and assuming the activating signal, in our case, IFN-α, is still bound to the receptor, STATs can be reactivated by phosphorylation via JAKs. This process continues until the activating signal is no longer available, or if an antagonistic molecule, such as the suppressor of cytokine signaling (SOCS), or the protein inhibitor of STAT (PIAS), whose expression is IFN dependent, interferes with the pathway. For our experiments, we used STAT-1 phosphorylation as a measure of IFN-α signaling as a result of treatment of healthy cells with medium collected from B virus exposed PBMCs. STAT1 can be alternatively spliced to generate two isoforms, STAT1α (91kDa) and STAT 1β (84kDa). The functions of the splice variants are not well known, but in the context of Type I IFN signaling, the variants appear to function similarly. STAT-1β lacks the transactivation domain, which would prevent the tethering of transcription factors after promoter binding, potentially directing a different gene expression profile than from that engaged as a result of STAT-1α. Lodige et al reported in a study of IFN-γ treated HeLa cells that STAT1β was not different than STAT-1α in the ability to bind to the JAKs, but that the nucleocytoplasmic shuttling of STAT-1β was slower, and that STAT-1β was more prevalent at lower levels of IFN-γ [214]. STAT-1 can undergo numerous post-translational modifications such as glycosylation, acetylation and phosphorylation all of which can influence the interactions with JAKs, other STATs, IRFs, ISGF promoter binding, transcription factor recruitment and IFN dependent gene expression. For our purposes, we used the phosphorylation of STAT-1 at tyrosine 701 as a marker of activation of STAT-1 and did not explore any other modifications.

To test our hypothesis that IFN-α from PBMCs does not activate the IFN-α signaling pathway we incubated human and macaque PBMCs with infected cell lysates containing B virus (MOI 5) for 24h. To confirm that the PBMCs could produce IFN-α, we added CpG 2216 at 5ug/ml to healthy PBMCs, which subsequently can induce IFN-α from pDCs in a TLR9 dependent manner. We used two negative controls for IFN-α production from PBMCs, medium only and MCL, in equivalent amounts to the B virus exposed PBMCs. We cultured with PBMCs with treatments for 24h and supernatants were collected by centrifug-
gation at 400xg for 10min. To determine if medium from the infected or control PBMCs (supernatants) could activate STAT-1 phosphorylation (p-STAT-1) in uninfected fibroblasts, RMFs and HFFs were grown to sub-confluency in 48-well plates and the cell-free supernatants transferred to uninfected fibroblasts. As a positive control for p-STAT-1, we treated uninfected fibroblasts with 50 U/ml of recombinant IFN-α. At specific times, medium was removed and cells were collected in 50ul of Laemmli buffer containing 2- β-mercaptoethanol and the samples were stored at -80°C. To determine if STAT-1 was phosphorylated, an SDS gel was run using 7.5% polyacrylamide, with equal loading by volume of the cell lysates (which was confirmed by using GAPDH as a loading control). Subsequently, the proteins were electro-blotted onto nitrocellulose (0.45 μm), blocked and then probed using anti-phospho (Y701)-STAT-1, total STAT-1, and GAPDH as a loading control. As shown in the left box of Figure 25, human fibroblasts treated for 1h with medium collected from B virus exposed PBMCs did activate-STAT-1 via phosphorylation, demonstrating that the levels of IFN-α were sufficient to initiate this pathway. As discussed previously, STAT-1 can be alternatively spliced to produce two isoforms, and HFFs exposed to medium collected from B virus exposed PBMCs resulted in the p-STAT-1α/β present at similar levels. Our positive control of CpG treated PBMCs, also induced p-STAT-1 α and β within 1 h post treatment, similarly to HFFs treated with medium from B virus exposed PBMCs. Although our MCL treated PBMCs failed to produced any detectable levels of IFN-α (Figure 21), we saw very weak p-STAT-1 α. Our positive control of IFN treated HFFs, resulted in activation of both isoforms but levels of p-STAT-1 α were greater. At 3 h following exposure to medium from B virus exposed PBMCs, HFFs showed a higher intensity band for p-STAT-1 α relative to what was observed at 1 h post treatment. The p-STAT-1 levels for MCL were unchanged at 3h and the IFN-α -treated fibroblasts showed a reduction in intensity of p-STAT-1 α, with the β isoform much weaker than observed in the samples collected at 1h. An interesting observation is that while all total STAT-1 levels were equivalent across samples, HFFs only expressed STAT-1α for total STAT-1. These results indicate that human PBMCs exposed to B virus can activate the IFN-α signaling pathway and that
B virus induces the expression of the STAT-1 β isoform. The major producer of IFN-α in PBMCs, are pDCs.

To compare the macaque response, as shown on the right box in Figure 25, macaque PBMCs exposed to B virus infected cell lysates were able to induce p-STAT-1 α in uninfected RMFs at 1h, and unlike humans did not induce the β isoform. Our negative control of MCL induced a low amount p-STAT-1 α and our positive control of CpG treated PBMCs induced low levels of p-STAT-1 α. RMFs treated with IFN-α induced both isoforms of p-STAT-1, although the α isoform was present at higher levels, where HFFs only induced the α isoform. There was an apparent delay the appearance of p-STAT-1, as the intensity of the band was greater at 3h post treatment than at 1 h for RMFs. The medium collected from CpG-treated PBMCs failed to increase the levels of p-STAT-1 at 1 h and only activated the α isoform. The IFN-α treated RMFs showed a greater intensity of the p-STAT-1 band at 3 h and additionally activated the β isoform. Interestingly, RMFs appear to have a basal level of STAT-1 β that is not present in HFFs as total levels of STAT-1 of both isoforms were equal. This is a crucial observation because while there are limited data on the functions of the different isoforms, it is clear that RMFs and HFFs respond differently to IFN-α stimuli, and treatment with medium from B virus exposed PBMCs resulted in differential activation of STAT-1 isoforms in HFFs and RMFs. While we have shown that both macaque and human PBMCs produce IFN-α in response to B virus (Figure 21), we have shown here that the IFN-α in medium from these activates the IFN-α signaling pathway in uninfected cells (Figure 25). We know that B virus replication is unaffected by medium collected from B virus exposed PBMCs (Figure 23), but the differential activation of STAT-1 isoforms between species will be explored in the near future as these differences may result in dramatic differences in IFN-dependent gene expression in each host. This differential effect will potentially inform differences in the pathogenesis of B virus infection in macaques and humans, and can be partially investigated in co-culture model systems in the absence of adequate small animal models. Data from such studies can provide insight into potential transgenic animals that may provide in vivo
models to study B virus infections with pathogenesis patterns similar to those observed in infected macaques or humans.

**Figure 25** Medium from PBMCs exposed to B virus activates Type I IFN signaling in uninfected fibroblasts

Fibroblasts derived from macaques and humans were cultured with medium collected from B virus exposed PBMCs for 1 and 3h. Fibroblast lysates were collected in 50 ul and run on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed for p-STAT-1 and total STAT-1, as well as for housekeeping protein GAPDH. Sup = supernatant (medium), MCL = mock cell lysate, BV: B virus, IFN= recombinant IFN-α 50U/ml, CpG= CpG 22165 μg/ml

3.9 Cell-culture medium from PBMCs co-cultured with B virus infected human or macaque fibroblasts activates Type I IFN signaling pathways in uninfected fibroblasts of each respective species

We showed previously that PBMCs co-cultured with B virus infected fibroblasts do not produce significant amounts of IFN-α and that co-culturing infected fibroblasts with medium from these PBMCs did not reduce B virus replication. To explore a possible mechanism of B virus resistance to IFN-α in fibroblasts in light of the observations that co-cultures failed to produce significant amounts of IFN-α, we hypothesized that medium from PBMCs co-cultured with B virus infected fibroblasts fails to activate the IFNαR signaling pathway. We predicted that medium from these co-culture models representing interactions in humans or macaques at the site of infection would fail to induce STAT-1 activation, or p-STAT-1, because the IFN-α levels were not significant when compared to mock cell lysate treated co-
cultures (Figure 21). To test this hypothesis, we infected fibroblasts with an MOI of 5 of B virus. At 1 hpi, we added PBMCs and incubated these co-cultures for an additional 23 h. The cell-free medium was transferred to uninfected fibroblasts and incubated for 1 h. Treated uninfected fibroblast lysates were prepared and analyzed by western blot assay to assess total STAT-1 and p-STAT-1 relative levels. As a negative control, medium collected from MCL treated PBMC co-cultures were used to ensure the IFN-α production and subsequent p-STAT-1 was due to virus, not to cell lysate. Two positive controls were used, one, which was CpG 2216 (5 μg/ml), to ensure the PBMCs in co-cultures could induce IFN-α, and the second positive control was Universal Recombinant Type I IFN, which was added to the uninfected fibroblasts to ensure that the fibroblasts were capable of activating the IFNαR signaling pathway. As shown in Figure 26, medium from human PBMCs co-cultured with B virus infected human fibroblasts activated STAT-1, indicating that the IFN-α released into medium from B virus infected co-cultures was sufficient to activate the IFN pathway. Our positive control for IFN-α production, CpG 2216, also activated STAT-1 in human cells, leading us to assume that IFN-α was the activator of STAT-1 in this experiment. We did see, however, that medium from mock cell lysate treated PBMCs co-cultured with infected fibroblasts activated STAT-1. As we showed in Figure 25, medium from PBMCs treated with mock cell lysate and co-cultured with B virus infected fibroblasts also produced IFN-α, which activated STAT-1, as confirmed in this experiment. Later studies, using a multiplex assay for 26 human chemokines and cytokines, showed that human PBMCs treated with MCL and co-cultured with B virus infected HFFs, produced significant levels of IL-6 (data not shown, M. Vasireddi), which is also known to activate STAT-1 B virus exposed human PBMC co-cultures also induced IL-6, but the levels were lower than those produced by PBMCs treated with mock cell lysates. The results from this experiment, indicate that the medium from human PBMC co-cultures with B virus infected human fibroblasts activate STAT-1, and thus the IFN-α pathway in uninfected human cells, but the activation is most likely due to the high amounts of IL-6 in the supernatants, not the low levels of IFN-α present in this particular treatment group.
The macaque response is quite different from the human response, in that the supernatants from macaque PBMCs co-cultured with B virus infected fibroblasts activated STAT-1 poorly. In contrast to humans, the supernatants from MCL treated co-cultures did not induce any STAT-1 activation leading to the conclusion that macaque PBMCs are not activated by cell lysates. The cell lysates in all of our experiments were produced using Vero cells, which were also used to produce all B virus and HSV-1 used in the experiments described in this dissertation. It is possible that since African Green Monkeys from which Veros are derived, are not sensed as foreign activating stimuli, because these Old World Monkeys are more closely related evolutionarily to macaques. To rule this out, additional experiments will be performed in the near future with virus and lysates prepared from human and macaque fibroblasts. But these results hint at the possibility that under the experimental conditions, macaque cells did not produce IL-6, but this will be evaluated again in additional experiments in the near future, not as part of this dissertation. Our data show that RMFs respond to IFN-α through the activation of STAT-1, albeit only weakly under the experimental conditions established. The positive control for IFN-α production from PBMCs, CpG 2216, led to only faint activation of STAT-1(Figure 26), which was not expected since our co-cultures with CpG 2216 induced greater levels of IFN-α than did B virus co-cultures (Figure 21). The faint bands for both B virus and CpG 2216 may be due to the short treatment interval, but additional experiments will be required to evaluate this further. In Figure 25, we showed that RMFs had a higher intensity band for STAT-1 at 3h than at 1h, indicating that the temporal control of the signaling pathway in RMFs may be different from that in HFFs. Because we measured STAT-1 activation at 1hpe by PBMC so-culture medium shown in Figure 26, we can hypothesize that the activation of STAT-1 would be increase at 3h.

The co-culture model system we have used for these experiments is a great tool for understanding how B virus infection modulates host immune responses at a cellular level, but these studies must be extended to evaluate multiple cell types and their interactions in a complex defense system. Our data provide for the first time knowledge that IFN-α is produced from pDCs, and that PBMCs can be used in
their place when reagents are lacking for the enrichment of pDCs in species, e.g., macaques and other nonhuman primates. We also now understand that the produced IFN-α activate IFNαR signaling pathways in human and macaque fibroblasts, each which are critical cells at the site of virus entry, but to what extent and how these pathways are modulated downstream remains to be investigated in future studies to unravel the impact of this pathway on the pathogenesis of natural and zoonotic infection.

Figure 26 Supernatants from PBMC co-cultures activate Type I IFN signaling in uninfected fibroblasts
Fibroblasts were cultured with supernatants from PBMC co-cultured with B virus infected fibroblasts for 1h. Cell lysates were collected in 50ul and run on a 7.5% polyacrylmide gel, transferred to a nitrocellulose membrane and probed for p-stat1. Sup=supernatant, MCL=mock uninfected mock cell lysate, BV: B virus, IFN=recombinant IFN 50U/ml, CpG=CpG 2216 5µg/ml

3.10 B virus infection of macaque and human fibroblasts does not activate or block Type I IFN signaling

In an effort to understand the resistance of B virus to the effects of IFN-α, derived from either PBMC supernatants, PBMC co-cultures or recombinant IFN-α treatment during low or high MOI infections across a range of IFN-α doses, we then hypothesized that B virus blocks the Type I IFN signaling
**pathway in fibroblasts.** The type I IFN receptor is activated upon binding by either IFN-α or IFN-β leading to the activation of JAK and STATs. We used the phosphorylation of STAT-1 as a measure of IFNαR ligation and signaling activation. In the first experiment, we addressed the question of whether B virus activated, either directly or indirectly, the Type I IFN pathway in RMFs or HFFs. We know that neither RMFs nor HFFs produce Type I IFNs during B virus infection, so we predicted that B virus infection in these cells does not result in the activation of STAT-1. To test this hypothesis, we infected fibroblasts in T25 flasks with an MOI of 5 for 24 h. We collected both the supernatant and infected cells at 0, 2, 4, 6 and 24h to measure both IFN-α production and STAT-1 phosphorylation (p-STAT-1). As predicted, at all times post-infection, B virus did not activate the type I IFN signaling pathway through p-STAT-1 in either RMFs or HFFs (Figure 27). As a negative control, we exposed fibroblasts to uninfected mock cell lysates, which also did not induce p-STAT-1. Additionally, we measured total-STAT-1 levels to ensure the presence of STAT-1 protein in B virus infected lysates and to confirm that B virus did not degrade STAT-1 (Figure 27).

**Figure 27 B virus infection does not activate Type I IFN signaling in human or macaque fibroblasts**

Fibroblasts were infected with B virus (MOI 5) in 24-well plates. Cell lysates were prepared and collected in 50ul Laemmli buffer and electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed for total STAT-1, p-STAT-1, and GAPDH. MCL=mock uninfected mock cell lysate BV: B virus MOI of 5.
Our data so far, indicates how B virus titers are not affected by IFN-α present in medium from PBMCs exposed to B virus infected lysates (Figure 23) or to recombinant IFN-α treatment (Figure 24). In an effort to explore the mechanism that B virus titers are not affected by IFN-α, we hypothesized that B virus blocks IFN signaling by blocking the activation of STAT-1 in fibroblasts. To test this hypothesis, we infected human and macaque fibroblasts in 24-well plates with B virus at an MOI of 5 and after 1 h adsorption we added 500 U/ml of recombinant type I IFN for 30 min. The cells were lysed and collected for analysis by western blot assay for the presence of total STAT-1, p-STAT-1, and GAPDH. As a negative control, MCL treated cells were used and for a positive control for p-STAT-1 activation, mock cell lysate treated cells were also treated with recombinant type I IFN (500 U/ml). The data from representative experiments, shown in Figure 28, indicate that B virus does not block the activation of STAT-1 by the added recombinant IFN-α, as levels were equivalent between MCL and B virus infected cells treated with IFN, but IFN-α is not produced in mock or B virus exposed fibroblasts. Recombinant IFN treatment increased total STAT-1 levels in both B virus infected cells and mock infected cells. GAPDH was used as a loading control and was equivalent across all samples. These data show that B virus did not block the activation of the type I IFN pathway in human or macaque fibroblasts. Previous data from our lab indicate that the interferon stimulated gene expression is not increased during B virus infection in HFFs [215], providing further confirmation of our results showing B virus resistance to IFN-α treatment. Based on these data, we can hypothesize that B virus blocks the IFN signaling pathway downstream of STAT-1 activation, and most certainly it is necessary to compare ISG expression in macaques because of the differential activation of STAT-1 isoforms.
Figure 28 B virus does not block the activation of the Type I IFN signaling pathway in macaque or human fibroblasts

Fibroblasts were infected with B virus at an MOI of 5 for 1h then treated with 500U/ml of IFN for 30min. Cell lysates were prepared and collected in 50ul of Laemmli buffer and run on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed for p-STAT-1, total STAT-1 and GAPDH. MCL=mock uninfected mock cell lysate, BV= B virus, +IFN w= 500U/ml IFN for 30 min.
4 CONCLUSIONS

B virus, a simplex virus closely related to HSV-1, is endemic in macaques of all species and is capable of causing fatal zoonotic infection in humans in up to 80% of cases, if not treated quickly enough after infection. Identifying the immune correlates of infection in both the natural and foreign hosts will allow us to develop therapeutics and prophylaxis against B virus infection. This dissertation aimed to address the defect in human immune responses to B virus infection, specifically the interferon response and the role(s) of pDCs during infection. Based on the observations that zoonotic B virus infection results in a delayed or ineffective immune responses and the knowledge of the importance of the IFN pathway in virus infections, we hypothesized: B virus modulates the IFN response during zoonotic infection by blocking plasmacytoid DC activation and subsequent IFN signaling pathways to circumvent host defenses, while these pathways remain intact in the macaque hosts.

The results from this dissertation disprove my hypothesis as shown by the data, for the first time, that human and macaque PBMCs produced similar amounts of IFN-α in response to direct B virus exposure, however, IFN-α production was significantly reduced when human pDCs (and PBMCs) were co-cultured with B virus infected fibroblasts as well as for macaque PBMCs co-cultured with B virus infected fibroblasts, as currently there are no reagents available for the isolation of pDCs from macaques. Further, both human and macaque fibroblasts continued to support B virus replication when treated with IFN-α, either from endogenous (B virus-exposed PBMCs) or exogenous (recombinant IFN) sources despite the activation of the IFN signaling pathway. We know that following B virus entry, virus replicates in dermal and epidermal layers of skin or mucosa. It has been shown by other laboratories, that limiting the amount of virus production at the entry site reduces the amount of virus that subsequently enters sensory nerve endings in the skin, thereby reducing the amount of virus that reaches the dorsal root or cranial ganglia sub-serving the area of entry. My studies were undertaken to identify whether there were differences between the responses of human pDCs and human and macaque PBMCs that
can migrate into the damaged tissues and whether/how these differences affected the amounts of virus produced.

Although the data presented in this dissertation disproved the hypothesis, these data contribute to the understanding of the pathogenesis of B virus in both natural and foreign host cells by showing that the IFN pathway is not differentially modulated in these hosts. Future studies are essential to understand the mechanism by which B virus is resistant to IFN because knowledge of this mechanism will direct the identification of therapeutic targets. We showed that immune cells produce IFN-α in response to B virus and that B virus does not block the activation of IFN-α pathways, therefore additional studies are needed to explore how this IFN influences the overall host response to B virus or if the B virus resistance to IFN is limited to cells isolated from primary sites of infection. IFN-α activates other innate and adaptive immune cells and may play a critical role in B virus pathogenesis in the nervous system as well as at peripheral sites. The delay or blockade of the production of neutralizing antibodies during ultimately fatal zoonotic infections is clearly one role that is distinctly different in humans and macaques. The impact of my findings must be studied further with respect to IFN-α activation of CD4 T cells and the resultant activation of B cells capable of producing these neutralizing antibodies. Additional studies are needed for understanding the mechanism of restricted production of IFN-α during co-cultures of both human and macaque cells, since this may be a target for modulation of immune responses within the foreign host. Activation of pDCs with viral DNAs, such as those likely released in infected cell lysates, may serve to increase the levels of IFN-α to activate key adaptive immune responses.
As a summary of this dissertation, our data support the model that if pDCs could gain access to infected peripheral skin via a bite or a scratch from an infected monkey, they would respond to B virus infected fibroblasts with the production of cytokines and chemokines. These cytokines would recruit and activate a number of immune cells, including monocytes, T cells, NK cells and other dendritic cells. Previous data from our lab showed that mDCs did not produce cytokines in response to B virus infected fibroblasts (**R. Abraham, M. Vasireddi et al., 2012, unpublished raw data), nor were NK cells functional as shown by their lack of production of perforin and granzyme (* M. Vasireddi et al., 2010, in preparation), indicating B virus specific modulation of immune responses. Furthermore, this dissertation has shown, that despite the production of cytokines, specifically IFN-α, B virus replication was not reduced in fibroblasts. These cytokines likely influence the microenvironment of immune cells in the infected skin, which may affect the accessibility of B virus to peripheral neurons or the rate at which it can enter, which may be more critical for survival against B virus infection than the levels of B virus replication at peripheral sites.
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To determine if different donors had a differential response to CpG, we isolated pDCs from two different donors on two different days. We also pooled the pDCs to determine if heterologous populations of pDCs would affect IFN-α production in pooled pDCs. pDCs were isolated from PBMCs and cultured with CpG at 10μg/ml for 24h. Supernatants were collected and analyzed for IFN-α production by ELISA. The concentrations of IFN-α differed between donors and differed by day in one donor. As expected, pooling of pDCs did not affect IFN-α, but instead was an average of the combined donor pDC IFN-α production.

Fibroblasts were seeded into 8 well chamber slides and grown for 2 days. Cells were fixed with methanol, blocked with 10%NGS and labeled with anti-vimentin (green; left), -fibronectin (green; center) and -cytokeratin (red; right). Nuclear stain was Hoescht (blue). RMFs were positive for vimentin and fibronectin, confirming fibroblast lineage but negative for cytokeratin, indicating a lack of keratinocyte contamination.
Figure 32 B virus kinetics in macaque and human fibroblasts
Fibroblasts were infected with B virus at an MOI of 10 in 24 well plates. Cell lysates were collected by scraping at 0, 1, 4, and 24hpi and stored at -80C. Samples were titrated by standard plaque assay. Red line=supernatants only, black lines=supernatants and cells. Error bars represent SEM.
Table 4 Raw data for B virus exposed pDCs of cytokine and chemokine production

Raw data for Luminex data presented in Figures 14 and X.15U=untreated, M=mock treated; B=B virus; H=HSV-1; 1, 2, 3 represent repeat wells. Black: below detection; blue: detectable range; red: above detection

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Table 5 Raw data for pDC cytokine and chemokine production in co-cultures

Raw data for Luminex data presented in Figures 16 and 17. U=untreated, M=mock treated; B=B virus; H=HSV-1; 1, 2, 3 represent repeat wells. Black: below detection; blue: detectable range; red: above detection.