Subversion of Natural Killer Cell Defenses Induced by a Deadly Zoonotic Virus

Mugdha Vasireddi
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SUBVERSION OF NATURAL KILLER CELL DEFENSES INDUCED BY A DEADLY ZOONOTIC VIRUS

by

MUGDHA VASIREDDI

Under the Direction of Dr. Julia. K. Hilliard

ABSTRACT

B virus (*Macacine herpesvirus 1, Cercopithecine herpesvirus 1, herpes B virus*) is an Old World monkey simplex virus endemic in macaques. B virus infection in its natural host, macaque, is very similar to HSV-1 infection in humans causing mild or asymptomatic infection. On the other hand, zoonotic infection in humans results in death in the absence of early initiation of antiviral drugs. Viruses evade host immune responses in order to survive and propagate. Most herpes viruses including HSV-1 down-regulate major histocompatibility complex class I (MHC class I) surface expression on infected cells in order to prevent CD8+ T-cell recognition and subsequent cell lysis. MHC class I molecules bind to the inhibitory receptors of NK cells and prevent NK cell activity. Thus, this mechanism protects HSV-1 infected cells from CD8+ T-cell lysis, making them sensitive to natural killer (NK) cell cytotoxicity. To investigate if B virus pathogenicity is a result of novel immune evasion mechanisms employed by B virus, we determined NK cell regulation during B virus infection. To this end, our experiments demonstrate that B virus does not down-regulate MHC I expression as effectively as HSV-1, leading us to hypothesize that B virus infected cells are resistant to NK cell activity. We examined the expression of MHC I chain related genes (MICA/ MICB), which are activation ligands to NKG2D receptors on NK cells. Our results
show that there is no significant difference in MICA and MICB expression between HSV-1 and B virus infected cells. Furthermore, we tested for the up-regulation of cytokines and chemokines responsible for NK cell activation and migration. Our results indicate a significant up-regulation of IFN-α from PBMCs co-cultured with HSV-1 infected cells, which plays an important role in activating NK cells. NK cells within these PBMCs up-regulate perforin release indicative of NK cell activity. PBMCs co-cultured with B virus infected cells do not up-regulate any cytokines or chemokines responsible for NK cell activity. As a result the NK cells within these PBMCs do not significantly up-regulate perforin release. These results demonstrate that B virus employs a novel immune evasion mechanism to subvert NK cell activity.

INDEX WORDS: B virus, MHC I, HLA-A, -B, -C, -E, -G, MICA, MICB, HSV-1, NK cells, PBMC, IFN-α and IP-10, Perforin, Granzyme B
SUBVERSION OF NATURAL KILLER CELL DEFENSES INDUCED BY A DEADLY ZOONOTIC VIRUS

by

MUGDHA VASIREDDI

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

2009
SUBVERSION OF NATURAL KILLER CELL DEFENSES INDUCED BY A DEADLY ZOONOTIC VIRUS

by

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Susanna F. Greer

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2009
DEDICATION

To my mom and dad for their constant support and unconditional love

To my mentor, Dr. Julia Hilliard for encouraging me to “seek the truth”

And in loving memory of my dear “Alex”
ACKNOWLEDGEMENTS

With all my heart, I thank Dr. Hilliard for her mentorship, support, and guidance. Her zest for science is a constant source of inspiration to me. Under her guidance, I learned to analyze and understand science with an open mind. She is the reason for the passion I developed for science and I will always revere her for instilling in me the value of keeping science pure.

I thank my committee members, Dr. Roberta Attanasio and Dr. Susana Greer for their advice and critical analysis of my research and for offering suggestions. I also thank Dr. Peter Krug for training me in BSL-4. I would also like to thank Dr. Ludmila Peryligina, Dr. David Katz, and Dr. Irina Patrusheva for their helpful suggestions during the course of my doctoral studies.

I thank my fellow students for their support and our bondage to one common goal. Thanks for keeping me sane through all the excitements and frustrations. I would like to take the opportunity to thank B virus research lab members, our bio-safety officer, diagnostic lab members, and the administrative staff for their kindness and support. I also thank Dr. Malcolm Zellars for giving me an opportunity to teach and improve my teaching skills. Special thanks to LaTesha for her support and patience that is parallel to none. I thank MBD program for funding my stipend.

Finally, I would like to thank my grand-father for his ideals, my parents for teaching me to love and enjoy everything I do and for believing in me, my sister and her family for always being there and looking out for me, and my dear friends from different stages of my life for their love and support.
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP Binding Casatte</td>
</tr>
<tr>
<td>ACV</td>
<td>Acyclovir</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cel</td>
</tr>
<tr>
<td>ara-A</td>
<td>Adenosine Arabinoside</td>
</tr>
<tr>
<td>β2m</td>
<td>β-microglobulin</td>
</tr>
<tr>
<td>BMBL</td>
<td>Biosafety in Microbiological and Biomedical Laboratories</td>
</tr>
<tr>
<td>BSL-4</td>
<td>Biosafety Level-4</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic Lymphocyte</td>
</tr>
<tr>
<td>DAP</td>
<td>DNAX Activation Protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>E</td>
<td>Early</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ERp57</td>
<td>Endoplasmic Reticulum Protein 57</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal regulated Kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FEAU</td>
<td>2'-fluoro-5-ethyl-Ara-U</td>
</tr>
<tr>
<td>FMAU</td>
<td>2'-fluoro-5-methyl-Ara-U</td>
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<tr>
<td>GCV</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage- Colony Stimulating Factor</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
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<tr>
<td>HFF</td>
<td>Human Foreskin Fibroblast</td>
</tr>
<tr>
<td>Hpi</td>
<td>Hours Post-Infection</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes Simplex Virus type 1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus type 2</td>
</tr>
<tr>
<td>HVP-2</td>
<td>Herpes Virus Papio- 2</td>
</tr>
<tr>
<td>ICP</td>
<td>Infected Cell Protein</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate Early</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol-1,4,5-Trisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor Tyrosin-based Activation Motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor Tyrosin-based Inhibitory Motif</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kapos's Sarcoma Associated Herpesvirus</td>
</tr>
<tr>
<td>L</td>
<td>Late</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated protein Kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
</tr>
<tr>
<td>MHC class I</td>
<td>Major Histocompatibility Complex Class I</td>
</tr>
<tr>
<td>MICA/B</td>
<td>MHC class I Polypeptide-Related Chain A/B</td>
</tr>
<tr>
<td>MK2</td>
<td>Macaque Kidney Epithelial Cells</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity Of Infection</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NK-T</td>
<td>Natural Killer T-cells</td>
</tr>
<tr>
<td>NKG2D</td>
<td>Natural Killer Group 2D</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid Dendritic Cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-------------</td>
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</tr>
<tr>
<td>PFU</td>
<td>Plaque Forming Unit</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphotidylinositol-3-Kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Peptide Loading Complex</td>
</tr>
<tr>
<td>PLCg1</td>
<td>PhospholipaseC gamma-1</td>
</tr>
<tr>
<td>PrV</td>
<td>Pseudorabies Virus</td>
</tr>
<tr>
<td>SHP</td>
<td>SH2 domain containing protein Phosphatase</td>
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<tr>
<td>SLP76</td>
<td>SH2 domain containing Leukocyte Protein of 76 KDa</td>
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<tr>
<td>SA8</td>
<td>Simian Herpesvirus Agent 8</td>
</tr>
<tr>
<td>SNK</td>
<td>Student Newman Keuls</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen Tyrosin Kinase</td>
</tr>
<tr>
<td>TAP</td>
<td>Transport Associated Protein</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine Kinase</td>
</tr>
<tr>
<td>TMB</td>
<td>TetraMethylBenzidine</td>
</tr>
<tr>
<td>TNF-a</td>
<td>Tumor-Necrosis Factor- Alpha</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella Zoster Virus</td>
</tr>
<tr>
<td>ZAP70</td>
<td>Zeta-Chain Associated Protein Kinase 70</td>
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</table>
1 INTRODUCTION

1.1 Part I. Literature Review

1.1.1 B Virus

1.1.1.1 Clinical Significance

Macacine herpesvirus 1 (Cercopithecine herpesvirus 1, Herpes B virus, B virus) is a simplex virus endemic to macaques. B virus belongs to the subfamily alphaherpesvirinae of the family Herpesviridae. Approximately 72% to 92% of the wild-caught monkeys of adult age were found to be B virus sero-positive in early field studies (49, 79, 86, 127, 177, 186). B virus in macaques causes mild or asymptomatic infection, unless the animal is immunocompromised. In most cases B virus mode of transmission between macaques is venereal (189). The manifestations of infection are similar to herpes simplex virus infection in humans resulting in life long infection with occasional oral (9, 84, 85, 130, 176) and genital lesions (177, 187). Stressors including immunosupression drugs (32), gang-caging (178, 189), transport, and climatic conditions (84) are associated with increased B virus shedding by macaques.

B virus infection in humans results in encephalitis, encephalomyelitis, and death in untreated patients. Approximately 30,000 macaques are imported each year in to the United States for biomedical research. B virus is a zoonotic virus, the only simplex virus that crosses species in nature, and as such, personnel working with macaques are at the highest risk of B virus infection. Approximately 50 B virus human cases are known in the U.S. biomedical community, of which many have been poorly documented. B virus is fatal in up to 80% of untreated
humans once the virus has reached the central nervous system (CNS). Early anti-viral intervention can help in preventing virus spread to central nervous system and reduce death rate to less than 20% as reported by the CDC B Virus Working Group (35).

B virus infection of non-macaque monkeys is usually fatal (38, 60, 77, 107, 179) and mostly observed when macaques have been co-housed with other monkey species. Virus can be transmitted from infected animals, from infected tissue (2, 13, 17, 69, 72), or in one case from human-to-human contact (31). Because B virus is fatal in foreign hosts such as, humans, it is important to understand the pathogenesis of zoonotic infection. Interestingly, B virus infection in most monkeys induces a strong, high-titered IgG response within 2-4 weeks of infection, however in humans B virus infection usually results in low levels of IgG, if any at all. Survivors of B virus infection generally show detectable levels of B virus specific IgG, and in some of these individuals these antibodies persist for decades, fluctuating significantly, suggesting periodic reactivation of latent B virus (unpublished results, National B Virus Resource Center, Atlanta, GA)

1.1.1.2 History of Herpes B virus

Dr. William Bartlet Brebner, a physician and research scientist, working on poliovirus was bitten by an apparently normal monkey on his fourth and fifth fingers. He developed ascending transverse myelitis and finally succumbing to infection following respiratory arrest. Autopsy results did not show any significant pathology. Gay and Holden examined some of the patient’s brain and cord tissue and prepared suspensions from these tissues for transmission experiments. They named this new virus “W” virus. Rabbits were inoculated intradermally with the above suspensions. These rabbits developed lesions characteristic of herpes viruses. When
rhesus macaques were inoculated with virus passaged in rabbits, they did not exhibit a severe disease. Thus, Gay and Holden concluded that this herpes-like virus is innocuous to rhesus macaques. Virus passaged in the rabbits was also used to infect cebus monkeys and the infection was fatal and characteristic of herpes infection in cebus monkeys. Finally, Gay and Holden conducted cross-immunity tests by using human and rhesus serum samples to neutralize a known herpes virus and also “W” virus. In their experiments both the known herpes virus and “W” virus behaved similarly. Thus, Gay and Holden concluded this “W” virus to be similar to other herpes viruses capable of causing encephalitis (60).

Subsequently, Sabin and Wright examined brain, spinal cord, spleen, and regional lymph nodes from the autopsy specimens and prepared emulsions of these tissues. They conducted transmission experiments using macaque monkeys. The first monkey that was infected by the tissue emulsions died without any apparent pathology. The tissue derived from this monkey was used to infect other monkeys, and these monkeys survived. The original brain and cord tissue from the human was then used to inoculate rabbits via intracerebral, and intracutaneous routes. The animals became infected and showed symptoms similar to humans and eventually died. From their experiments, Sabin and Wright reported the causative organism to be a filterable agent, as did Gay and Holden. They noted that this filterable agent was similar to human herpes simplex virus, but noted further that this agent had properties that would allow classification of it as a new and a different virus from human herpes simplex virus. This virus was named “B virus” by Sabin and Wright to honor the first known victim who was an accomplished scientist and who has contributed much to science (39) (6). That name has subsequently been used from that time forward.
1.1.1.3  

B virus growth and replication

B virus is currently regulated as a Select Agent by the U.S. Department of Homeland Security and in accordance with the 5th Edition of the bio-safety in microbiological and biomedical laboratories (BMBL) (166) and the propagation of B virus can only be done in a bio-safety level-4 (BSL-4) laboratory. B virus propagation in a tissue culture based system was first demonstrated by Burnet et al., using chorioallantoic membrane of the developing chick embryo (28). Reissig et al., infected adult rhesus kidney epithelial cells with B virus at multiplicity of infection (MOI) 2 from virus stock prepared and titered in monkey renal tissue culture. After 2 hours post-infection (hpi), infected cells contained vacuoles and more significant changes started to appear at 4-6 hpi. Between 4 and 6 hpi, irregular and larger nucleoli were observed. Clear changes in chromatin occurred by 10 hpi and by this time new virus particles started appearing. Both intracellular progeny virus and extracellular virus titers start increasing at 6 hpi and keep increasing until 24 hpi, after which the virus titers plateau. At 18 hpi, polynucleated cells were observed and these cells were formed due to cell fusion, called syncytia (148). Around the same time, Krech and Lewis passaged B virus obtained from an infected monkey in rabbits. The tissue fluid obtained was used to infect the chick embryos and monkey kidney cells (derived from rhesus or cynomolgus macaques) (89). Their stability studies show that B virus is stable for 8 weeks when stored at 4°C, however, B virus loses its infectivity by 2 logs when stored at −20°C and −72°C (89). Subsequently, the kinetics of virus replication and the identification of infected cell polypeptides was published (70). B virus replication kinetics in cell culture was similar to herpes simplex virus type 1 (HSV-1) replication kinetics.
In the natural host B virus replicates at the site of infection and travels by retrograde transmission and establishes latency in the dorsal root of the sensory ganglia. B virus reactivation is thought to be stimulated upon exposure to stress factors such as UV exposure, hormonal stress, infection and other unknown factors. Virus travels anterograde, returning to the epithelial site where it was initially introduced (78) (50), (24), (64). Thus, B virus life cycle in a natural host, macaques, is similar to HSV-1 life cycle in natural host, humans. In humans, however, B virus spreads to the CNS ultimately affecting the integrity of the brain stem (75). Respiratory arrest results, although it remains unknown whether this is due to virus or immune response.

1.1.1.4 B virus structure and genome

B virus size and structure is similar to that of other herpes simplex viruses, such as, HSV-1 and herpes simplex virus type 2 (HSV-2) (111). It has a glycoprotein studded envelope within which is the tegument surrounding a nucleocapsid that contains the circular double stranded genome of approximately 156K base pairs rich in G + C content. Electron microscopy studies of B virus have revealed that the virus is transported from cytoplasm to nucleus at relative earlier time points than HSV virions. The enveloped virus within the cell was found to be located near the perinuclear spaces, endoplasmic reticulum (ER), and Golgi complex. B virus was described by Ruebner et al. to exit through the cytocavitary network rather than through microfilaments as suggested in the case of HSV-1 (154). These studies have never been expanded upon to date.

Previous data from Harrington et al., predicted the size of B virus genome to be 165.2 Kbp (92). Complete genome sequence analysis of B virus lab strain E2490 by Perelygina et al., demonstrated that B virus genome is 156.789 Kbp in length, consisting of approximately 75%
G+C content. The G+C content in B virus was found to be significantly greater than HSV-1 and HSV-2 (137), and perhaps the highest of any simplex virus. The B virus genome when compared to that of HSV-1 has >80% DNA sequence homology (92, 137). Also, a high DNA sequence homology is observed between HSV-1, HSV-2 (human herpes simplexviruses), B virus, HVP-2, and SA8 (non-human primate herpes simplexviruses) (81).

### 1.1.1.5 Animal model systems for B virus

The first successful animal model for B virus studies was a rabbit model, used by Gay and Holden and later by Sabin and Wright to investigate the first documented zoonotic infection. Rabbits were injected with tissue-collected post-mortem with B virus infection. The tissue was used to inoculate the rabbits via intracerebral, intracutaneous, intratesticular, and intraperitoneal routes. Infection through intracerebral route resulted in convulsions, salivation, and death within 24 hpi. While intracutaneous routes resulted in flaccid paralysis and respiratory failure followed by death 6 days post inoculation with onset of symptoms appearing by 24 post inoculation with cutaneous lesions. Intratesticular infection resulted in paralysis and ascending myelitis 7 dpi. Complete posterior paralysis followed by death on 11 day was observed after the animal was infected through intraperitoneal route. Therefore, Acute B virus infection in rabbits was very similar to that observed in humans (6) (60).

Later in 1954, Melnick et al., published their results from experiments in which various animals such as, cotton rats, albino mice, guinea pigs, rabbits, and hamsters were inoculated with rhesus macaque poliomyelitis infected tissue. None of their experimental animal models showed any signs of poliomyelitis as per the observations from pathological and histological studies. For their transmission experiments, brain suspension of a rhesus macaque was inocu-
lated into cotton rats and the infected CNS tissue from cotton rats was passaged several times. The infected tissue obtained from the final passage in cotton rats was used to inoculate different animals in order to study the B virus host range. When they infected four-week old cotton rats via intracerebral and intraperitoneal routes of inoculation, they observed myelitis, perivascular infiltration, neurodegeneration, and encephalitis in examined spinal cord sections. They observed no apparent damage to the meninges. Intracerebral inoculation of infected tissue into newborn mice resulted in ataxia, failure to grow, abnormal movements and ultimately leading to death. Intracerebral inoculation of 4-week-old mice resulted in meningoencephalitis and infiltration of lymphocytes into the brain. Intracerebral routes of inoculation of infected tissue into guinea pigs resulted in development of meningitis, however, subcutaneous inoculations caused necrosis around the site of inoculation. Inoculation tissues into rabbits yielded similar results to those observed by Sabin and Wright. Intracerebral route of inoculation of tissue into hamsters resulted in ataxia, irregular movements, and prostration followed by death. Each of these symptoms was consistent with the symptoms noted by Sabin and Wright in their transmission experiments of rabbits that were injected with B virus infected tissue (115). Thus, these animals were infected with a virus that was innocuous to macaques and later classified and named B virus.

Many decades later, Richey et al., infected mice with B virus and reported that symptomology was similar to that observed in humans with some B virus isolates, but that each isolate differed in terms of ability to cause symptoms and doses at which significant numbers of animals showed apparent signs of infection. Severity of infection was clearly B virus strain- and dose-dependent (149). In these experiments the investigators inoculated BALB/ c mice intra-
muscularly with virus preparation of different serial dilutions. Similar experiments in a different strain of mice (CS7BL/6) did not result in a different outcome from that observed in BLAB/c mice once disease in CNS is observed (149). This study emphasizes on the fact that B virus virulence in a foreign host is a result of many factors such as, virus strain, viral dose, and host immune responses against the invading virus.

1.1.1.6 B virus diagnosis and treatment

B virus can be diagnosed by isolation of virus or identification of antibodies. In some cases virus isolation is difficult if not impossible and reliance on clinical symptoms and/or the presence of increasing antibodies is the basis of diagnosis and treatment. B virus-specific PCR can be used additionally, but this technique does not confirm the presence of replication competent virus. (110, 112). Because there can be antibody cross reactivity if the person has antibodies against herpes simplex viruses (1, 40, 83), competition ELISA and western blot assays must be used for serodiagnosis.

Nucleoside analogs such as acyclovir (ACV), ganciclovir (GCV), and adenine arabinoside (ara-A) are used against HSV-1 and HSV-2 infections in humans. ACV was also found to be effective against other human herpes virus infections such as varicella zoster-virus (VZV), human cytomegalovirus (HCMV), and Epstein barr virus (EBV) (36, 52). ACV is phosphorylated to triphosphates by viral thymidine kinase (TK). Once phosphorylated, ACV inhibits viral DNA polymerase and ultimately viral replication (146). 2`-fluoro-5-substituted arabinosyl pyrimidine nucleosides such as 2`-fluoro-5-methyl-Ara-U (FMAU) and 2`-fluoro-5-ethyl-Ara-U (FEAU) were found to be effective against human herpes simplex virus at the same time were less toxic to the cells in cell culture (87, 113). Zwartouw et al, compared ACV and GCV for treatment of B
virus in rabbits. They found that GCV was more effective than ACV even at lower doses, however, GCV was not found to be as effective if administered later during infection (188). Therefore, ACV (10 mg/kg) is an effective anti-viral used in the treatment of B virus infection in the absence of symptoms in humans. Prophylactic treatment with acyclovir can also be effective, since it may inhibit replication of B virus at peripheral sites, reducing the virus load available for establishing latency in subserving sensory ganglia. This may in turn reduce the probability of reactivation of B virus in individuals who survive infection (25, 75). Recent studies by Krug et al, have shown that FEAU and FMAU nucleoside analogs were 100 times more potent than conventional nucleoside analogs such as ACV and GCV (90). Therefore, FEAU and FMAU could potentially be used as “compassionate use” drugs for the treatment of advanced zoonotic infection.

Attempts to develop vaccines to prevent B virus have not been successful in macaques (16, 108). In order to produce vaccines to induce good immune response, it is important to understand how B virus interacts with host immune responses and what the immune correlates of infection are. Interestingly, although HSV-1 infection in humans induces specific and detectable adaptive immune response, zoonotic B virus infection fails to induce a detectable adaptive immune response comparable to that seen with HSV infection. In each of the B virus fatal cases, at least since 1987, infected individuals failed to show robust antibody production against B virus.

HSV-1, which is also a herpes simplex virus evades specific immune responses in order to survive in a natural host. B virus is very similar to HSV-1 in its life cycle in a natural host (60) sharing extensive homology with HSV proteins (137), a point which suggests that B virus may have selected similar strategies for evading host immunity. Improving understanding of the
immune correlates of B virus infection in the natural versus the foreign host may illuminate how to redirect the immune response in infected humans such that morbidities and mortalities may be avoided in cases of zoonotic B virus infections.

1.1.2 Immune evasion mechanisms by herpesviruses

Within natural hosts, viruses successfully replicate and spread without adversely affecting the host. This guarantees the availability of the host for virus. A host, in turn, will elicit strong immune responses to stop or minimize the effects of the virus invasion, however, viruses select traits that facilitate bypassing restrictive host immune responses. As long as a balance is maintained between virus propagation and host immune responses, virus will be able to propagate in successfully and immune responses will protect the host against morbidity and mortality.

Of the many evasion mechanisms, down-regulation of surface expression of major histocompatibility complex (MHC class I) is hallmark of all herpes viruses. Cellular immune responses involve the activities of CD8+ T-cells, cytotoxic lymphocytes (CTL) that recognize foreign antigen presented by the MHC class I molecules on the infected cell surface. Antigens presented are the result of proteosomal degradation of foreign proteins to 8-10 amino acid long peptides. The US12 gene of HSV-1 and 2 encode for Infected cell protein 47 (ICP47), an immediate early (IE) protein, which binds to the cytosolic transport associated protein (TAP) involved in the transport of peptides to the MHC class I molecule in the endoplasmic reticulum (ER) (57, 68, 184). This blockade of peptide translocation by HSV ICP47 results in the sequestration of MHC class I in the ER. The unloaded MHC class I is degraded via ER degradation pathway, which will result in absence of MHC class I on the cells surface. Thus, the sequestration of MHC class I in
the cytoplasm inhibits the HSV specific CTLs from recognizing the infected cell and killing them (59, 61, 80, 93, 123, 124, 164, 165). Although HSV specific CTLs are generated due to antigen presentation by antigen presenting cells (APC), these CTLs are unable to lyse HSV-1 infected cells due to lack of MHC class I on the cell surface. Thus, the presence of HSV antigen specific to CD8+ T-cells is important for limiting the spread and reactivation of the virus and not so much during primary infection (129, 171).

The critical 3-35 amino acid sequence of ICP47, which binds to the peptide-binding region of TAP and inhibits peptide loading, is conserved in both HSV-1 and HSV-2 (63, 122, 140, 153, 164). This TAP binding domain is absent in each of the other alphaherpesviruses studied to date, although most of the known alphaherpesviruses, such as, pseudorabies virus (PrV), varicella zoster virus (VZV), equine herpesvirus-1, and bovine herpesvirus-1 down regulate MHC 1 (180). PrV is similar to HSV-1 in down-regulating MHC class I, in that, peptide transport is blocked and the MHC class I remains unloaded (8). During VZV infection, MHC class I is accumulated in the Golgi complex and prevented from surface expression (4). Equine herpesvirus-1 down regulates MHC class I surface expression by blocking peptide translocation via TAP (7, 143). Bovine herpesvirus 1 UL49.5 blocks TAP dependent peptide translocation, thus down regulating surface MHC class I (88). Non-human primate simplex viruses, such as B virus, herpes virus papio-2 (HVP-2), and simian herpes virus (SA8) contain US12 gene encoding ICP47, however, the TAP binding sequence present in HSV-1 and 2 ICP47 is absent (19). The down-regulation of MHC class I on the cell surface of an infected cell results in sensitivity to natural killer (NK) cells lysis. NK cells are also cytotoxic lymphocytes, but are not specific in the sense that they only distinguish between self and non-self antigen. NK cells recognize a self-antigen
presenting MHC class I and are thus inhibited from killing the cell. When present on the cell surface, MHC class I molecules bind to the cell surface inhibitory receptors on NK cells, thus acting as inhibitory ligands. In case of certain virus infections, such as herpesviruses where MHC class I is down regulated, these cells become sensitive to NK cell lysis. Certain herpes viruses like CMV employ mechanisms to down regulate NK cell recognition of infected cells. This mechanism is not observed in the case of HSV-1 and HSV-2 thus resulting in lysis by NK cells. **The mechanism of regulation of MHC class I and the subsequent modulation of NK cell activity during B virus infection is the major focus of this thesis.** Later in this section, details of MHC class I presentation and NK cell regulation will be discussed.

Another sub-type of CTL, which is far less studied but as important as CD8+ T-cells are natural killer T-cells (NKT). NKT cells are a subtype of CTLs that recognize glycolipid antigens presented by CD1d molecules on the cell surface. Viruses can block lipid presentation to evade NKT cell activity (170). NKT cells express both CD8+ T-cell and NK cell markers and thus the name NKT. During HSV-1 infection, presentation of CD1d molecules on the cell surface is blocked by displacing CD1d through endocytosis (185). The virus protein responsible for this mechanism is not known currently. Thus, this is another example of how HSV-1 and HSV-2 employ different mechanisms to delay or prevent immune responses that can block successful replication and spread of the virus.

1.1.3 **Major Histocompatibility Complex Class I (MHC class I)**

MHC class I molecules are constitutively expressed on the cell surface in all cells except red blood cells, and these are tightly regulated in neurons. MHC class I expression is enhanced by the exposure of cytokines such as, tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1),
interferon-gamma (IFN-γ), and interferon-alpha (IFN-α). Genes encoding MHC 1 are regulated by transcription factors belonging to Rel family members including nuclear factor kappa-light-chain-enhancer or activated B cells (NF-κB). These factors bind to the enhancer region of the genes upstream of initiation region at the κB binding site. H2TF1, which was found to share sequence homology with many members of Rel family members, is implicated as the major transcription regulator of MHC class I (141). Thus, H2TF1 is an ideal target for many pathogens and tumor cells to down regulate surface expression of MHC class I (102).

MHC class I molecules are composed of a transmembrane heavy chain that has three domains: α1, α 2, and α3. Domains α1 and α 2 are the peptide binding domains (125, 128) and α3 domain serves to anchor the molecules into the membrane of ER (131). MHC class I molecules must be folded properly for stability in order to be expressed on the cell surface. The proper folding of MHC class I in the ER is achieved by the association of calnexin, a transmembrane protein, to MHC class I molecule (65). Once MHC class I molecules bind to β2 microglobulin (β2m), calnexin disassociates from the MHC class I protein (125, 128, 160). The MHC class I heavy chain with β2m is further stabilized by another transmembrane protein calreticulin, which recruits endoplasmic reticulum protein 57 (ERp57) (48, 56, 100). Residue Cys-57 of the α domain of ERp57 forms a disulfide bond with Cys-95 of tapasin (12, 43), thus forming a calreticulin/ERp57/ MHC-1/ β2m/ tapasin complex on the ER membrane. This complex is important for peptide loading and is called MHC class I peptide loading complex (PLC) (136). Interaction of TAP1 and TAP2 with MHC class I PLC is mediated by tapasin. The TAP protein belongs to ATP binding cassette (ABC) family of transporters. Approximately 8-10 amino acid peptides bind to the peptide-binding domain of TAP to be transported to the MHC class I peptide-
binding domain. This process of peptide loading by TAP to the PLC is an energy consuming process involving hydrolysis and translocation of ATP (3, 136). Another protein ER aminopeptidase associated with antigen processing (ERAAP) trims the peptide to an appropriate length to be loaded onto MHC class I complex (155, 158, 183).

The 20S subunit of the proteosome is involved in degradation of proteins to peptides that can be loaded on MHC class I peptide-binding domain via TAP. 20S subunit together with 19S (PA700) forms the 26S proteosome involved in the degradation of large polyubiquitinated and mis-folded proteins (42). The base of 19S has 6 ATPases that are required for the unfolding of proteins, however the degradation of proteins by 20S proteosome itself is an ATP-independent process mediated by 11S regulatory complex (PA28) (32, 47, 131, 147). Peptides that bind to MHC class I are 8-10 amino acids long and contain a hydrophobic C-terminus. The peptide-binding site present on TAP1/TAP2 hetero-dimer suggests an equal role for both proteins in peptide binding. In uninfected cells, self-proteins are degraded to MHC class I specific peptides by the proteosome and loaded onto the MHC class I complex. Once the MHC class I molecule is loaded with peptide, it disassociates from the chaperone complex and is transported to the cell surface through the secretory pathway where the MHC class I is further glycosylated and modified in Golgi complex and secretory vesicles. These modifications are necessary for MHC class I protein stability and proper antigen expression on the cell surface. MHC class I proteins that are not properly folded or stabilized will undergo degradation in the ER through ER-aminopeptidate associated antigen degradation pathway (ERAAP) (136). The above process of MHC class I class I antigen presentation is illustrated in Figure 1.
During infection, virus proteins are degraded by proteosome, loaded onto the cell surface to be recognized by specific CD8+ T-cells. Because self-reactive T cells are destroyed during thymic selection processes, normal cells presenting self-antigen are not destroyed by CD8+ T-cells. To minimize presentation of foreign antigen and thus cytolysis, many viruses down regulate MHC class I expression on the cell surface by disrupting MHC class I peptide loading and/or transport to cell surface, which is illustrated in Figure 2.
Figure 1: MHC class I antigen presentation on the cell surface. Proteins are degraded by proteosome into 8-10 aa peptides, which are loaded on MHC class I molecule via TAP. The MHC class I molecule disassociates from the chaperone complex and translocates to surface through the secretory pathway.
Figure 2: Viral down-regulation of MHC class I on cell surface. Different viruses block MHC class I expression the cell surface at different stages of antigen presentation the cell surface.
There are 6 isoforms of MHC class I of which HLA-A, -B, and -C are categorized as MHC class Ia and HLA-E, -F, and -G are categorized as MHC class Ib. MHC class Ia molecules are generally involved in antigen presentation to CD8+ T-cells, however they also function as inhibitory ligands to NK cell receptors and inhibit NK cell activation. HLA-E and HLA-G predominantly function as NK cell inhibitory ligands, but the function of HLA-F is not known currently. HLA-E is present in as much abundance as HLA-A, -B, and -C. There are two transcripts of HLA-E, short and long transcripts, regulated by alternative polyadenylation. Short transcript is found slightly more in cancer cells than long transcript, otherwise there are found in same amounts with the long transcript probably being the functional one (167). HLA-E expression is regulated by CIITA transcription factor and is responsive to IFN-γ (30). HLA-E associates with β2m and binds to nonamers of HLA-1a signal sequence-derived peptides obtained by proteosomal degradation (22). TAP hetero-dimer is required for stability and peptide binding to HLA-E. Thus, a non-functional TAP hetero-dimer will lead to down-regulation of HLA-E surface expression (101). In contrast, recent studies on HLA-E have shown that in the absence of signal sequence-derived peptides, a functional TAP or tapasin results in surface expression of an immature HLA-E glycoforms. (104). Stable HLA-E molecules on the cell surface are available to bind to NK inhibitory receptor CD94/NKG2A (23, 101). CD94/NKG2A complex binds at the peptide-binding domain α1 and α2 heavy chain of HLA-E (82). Not all HLA-E molecules that bind to signal sequence-derived peptides bind to CD94/NKG2A inhibitory receptors. HLA-E that bind to only specific peptides derived from some isoforms of HLA-1a are capable of binding to NK cell inhibitory ligand and inhibition of NK cell cytotoxicity (54, 101).
HLA-G protein is selectively expressed on placenta and thymus (76). The transcription of HLA-G is not dependent on NF-κB, CIITA, or IRF-1, but is regulated by RFX and SP1 transcription factors, which differs from HLA-1a or HLA-E transcription regulation (30). Also, IL-10 cytokine induces HLA-G cell surface expression especially in cancer cells (157, 168). HLA-G exists in 7 isoforms, HLA-G1, -G2, -G3, -G4, -G5, -G6, and -G7, as a result of alternative splicing. HLA-G1, -G2, -G3, and -G4 are membrane bound while HLA-G5, -G6, and -G7 are soluble forms. HLA-G is differentially expressed in different cells (30, 169), (29), (116). The structure of HLA-G is very similar to each of the other HLA molecules consisting of a heavy chain with 3α domains, and with α1 and α2 forming the peptide-binding groove. The third domain binds non-covalently to β2m. HLA-G can present peptides in a TAP dependent or TAP independent manner (34). HLA-G isoforms expressed on the cell surface bind to variety of NK cell inhibitory receptors such as, leukocyte Ig-like receptor (LIR-1) (62) and KIR2DL4 (NK cell killer inhibitory receptor). HLA-G2, -G3, and -G4 isoforms not only block NK cell lytic activity (135, 150, 151), but also block CD8+ T-cell lytic activity (152). HLA-G dimerization can occur in some instances on the cell surface. The cell surface dimerization occurs due to the disulfide bonds of Cys-42 of the heavy chain (26).

1.1.4 Natural killer cells

Natural Killer cells (NK cells) are lymphoid origin cells that play an important role in both innate and adaptive immunity. NK cells are mainly known to be part of innate immune responses, however, recent studies have shown data that suggest NK cells share not only similar phenotypic features with T-cells but also, certain functional features such as, cytotoxicity, memory, proliferation, and contraction (41, 161, 182). There are two subsets of NK cells: CD56bright / CD16dim and CD56dim / CD16bright. About 90% of NK cells are CD56dim and respond to
IL-2 playing an important role in cytotoxicity including antibody dependent cell cytotoxicity (ADCC). The other 10% of NK cells, which form the CD16^{bright} population, upon activation produce IFN-γ, TNF-α, and granulocyte macrophage colony-stimulating factor (GM-CSF) as well as other cytokines (IL-10 and IL-13) that drive a Th2 immune response. Each subset is capable of similar cytolytic activity (51).

NK cells can lyse infected or cancerous cells in the absence of antigen presentation, however, presence of proper activation ligands on the surface of target cells is important for activation of NK cells. As discussed previously, under normal conditions, cells present self-antigen via MHC class I pathway. These are recognized as “self” by NK cells as they bind to the MHC class I molecules and are inhibited from lysis. When cells are infected with viruses that down regulate MHC class I, NK cells are not inhibited due to “missing self” or inhibitory ligands. The presence of activation ligands will activate the NK cell to kill the infected cell lacking MHC class I. Thus, MHC class I molecules act as inhibitory ligands that bind to inhibitory receptors present on the NK cells (95, 96, 98, 117, 181).

There are various activation receptors on NK cells and they fall in three main categories: ITAM (immunoreceptor tyrosine based- acvtivation motif), non-ITAM, and integrins (27, 97, 175). While most inhibitory receptors bind to MHC class I molecules, activation receptors of NK cells are more diverse, facilitating activation of NK cells in different situations, e.g., when cells are infected with either a pathogen that regulates specific ligands or when cells are transformed. FcgIIIR receptor belongs to ITAM activation receptors where the FcgIIIR (CD16) binds to IgG and results in ADCC (174). Certain activation receptors also bind MHC class I molecules. For example, KIR2DS1-2 is an ITAM NK cell activation receptor that binds to HLA-C, however for
KIR2DS1-2 to activate NK cells it dimerizes (98). Another ITAM activation receptor NKG2C binds to HLA-E ligands, because of its homology to NKG2A inhibitory receptor that also binds to HLA-E. Receptors KIR2DL4 and BY55, non-ITAM activation receptors, bind soluble HLA-G and HLA-C, respectively. How and when the HLA-A molecules selectively bind to activation or inhibitory receptors is not well defined. Number of these activation and inhibitory receptors are also present on CTLs.

Certain activation ligands are expressed at basal levels on specific cell surfaces, however, these activation ligands in the presence of MHC class I molecules are insufficient to activate NK cells, thus, preventing cytolysis. During stress such as, infection or tumorogenesis, cells over-express specific activation ligands, thus activating NK cells even in the presence of MHC class I molecules. The molecule NKG2D is a non-ITAM activation receptor on NK cells that binds to MICA, MICB, or ULBP (1-4) ligands expressed on the target cell surface. NKG2D is also expressed on T-cells. These NKG2D ligands belong to MHC class I family where the protein folding is very similar to MHC class I. The binding of ligands to NKG2D receptor can override the inhibitory effect of MHC class I when present (15, 37, 44, 45, 106).

Certain viruses, e.g., many herpesviruses, down regulate surface MHC class I thus making infected cells sensitive to NK cell–mediated cytolysis. The beta-herpesvirus, HCMV (cytomegalovirus) down regulates MHC 1, however, peptide from the leader-sequence of HCMV UL40 glycoprotein binds to HLA-E and up-regulates HLA-E expression independently of TAP (163). This HLA-E presenting UL40 peptide binds to the NKG2A/CD94, protecting the infected cell from NK mediated cytolysis. Additionally, CMV UL16 binds to ULBP stress proteins and down regulates their surface expression. CMV also expresses specific proteins that mimic NK
cell activation ligands and disrupt NK cell activity. With these mechanisms in place, CMV can
down regulate both MHC class I expression for CD8+ T-cell presentation (133) and also NK cell
activity (activation and inhibition of NK cells is illustrated in figure 3), sparing the infected cells
so that virus replication can proceed unimpeded.
Figure 3: NK cell activation and inhibition. A normal cell, which expresses MHC class I and basal levels of activation ligands, is resistant to NK cell lysis. In cases of over expression of activation ligands or down-regulation of MHC class I results in NK cell lysis of target cells.
Within the NK cell, ligation of ITAM receptors recruits DAP12 adaptor molecules, an event which is followed by phosphorylation of Syk/ZAP70 tyrosine kinases. This phosphorylation event leads to further downstream signaling events that induce mitogen activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK), a pathway resulting in release of cytotoxic granules and/or expression of cytokines IFN-γ and TNF-α, GM-CSF, IL-2, and other Th2 associated cytokines. When ligation of non-ITAM receptors e.g., NKG2D occurs, DAP10 adaptor molecules are also recruited, but the signaling pathway is independent of Syk phosphorylation. In these cases, the signaling pathway involves phosphorylation of phosphatidylinositol 3 kinase (PI3K) and phospholipase C-γ1 (PLCγ1) (20, 97, 99, 138, 175). Upon ligation of inhibitory receptors by MHC class I molecules, SHP1/2 protein is phosphorylated, preventing phosphorylation of SLP-76 and Vav proteins, each of which is involved in ITAM and non-ITAM induced activation (172, 173, 175) (Figure 4). For NK cells to properly function, they purportedly adhere to the target cells. The adherence is mediated by the integrin receptors on the NK cells when these bind to ligands, e.g., ICAM, VCAM, and fibronectin. This ligation facilitates lysis of target cells (27). Alternatively, NK cell killing may be facilitated by exocytic release of lysosomes containing granzyme and perforin obviating the need for direct cell-to-cell contact.
Figure 4: NK cell inhibitory and activation signaling. Inhibitory ligation results in the phosphorylation and activation of SHP, which leads to dephosphorylation of SLP76 and Vav inhibiting NK cell activity. Activation ligation through ITAM recruits DAP12 and phosphorylation of Syk and Zap70 leading to NK cell activity. Activation ligation through non-ITAM recruits DAP10 and phosphorylates PI3K to activate NK cell activity.
In order for NK cells to bind to respective ligands, NK cells need to be primed and recruited to the site of infection or cancer growth (localized tissue). This activation and migration is induced by certain cytokines, which are produced by the infected cell. Type II cytokines, e.g., IFN-γ are involved in NK cell migration and cytotoxicity activity. Likewise, IL-2, IL-12, TNFα, IL-1α/β, IL-15, and IL-10 are all important for production of IFN-γ production by NK cells upon activation. Beside these cytokines, chemokines such as, MIP-1α, MIP-1β, IP-10, and MCP-1, 2, 3 are involved in the chemoattraction, migration, and activation of NK cells (21, 162).

NK cells upon activation exert effects on target cells in two ways: cytotoxicity and/or cytokine production. Cytotoxicity is mediated by release of perforin granules and granzyme protease molecules from the NK cells via exocytosis after engaging the target cells or by ligation of TRAIL and FAS receptors. Cytokines such as, TNF-α and IFN-γ produced by activated NK cells result in apoptosis of target cells. IFN-γ, a Th1 cytokine, produced by NK cells can result in activation of CD8+ T-cell cytotoxicity (121). NK cells also produce, although in smaller amounts, Th2 cytokines (66, 67, 91) and play a role in regulating hematopoiesis and B-cell differentiation (10, 139). NK cells can interact with DCs to activate them and help antigen presentation. This is a bidirectional interaction, where dendritic cells can activate NK cells by production of IFN-α. Thus, NK cells and dendritic cells exert influence upon each other either by the production of cytokines or by receptor-ligand binding (145). Thus, NK cells upon activation can modulate not only innate immune responses, but also the adaptive immune responses and act as a bridge between innate and adaptive immune responses.
1.1.5 Natural Killer cells and encephalitis

Natural killer cells are associated with the innate immune responses, but also play a major role in adaptive immune responses. Because of these important roles, NK cells are implicated in infections leading to encephalitis. Aforementioned, NK cells can induce the production of Th1 and Th2 cytokines that affect the T cells to produce cellular and/or humoral immune responses. NK cells play a major role in providing protective immunity against various encephalitis-causing viral infections, such as, HSV-1, rabies and Theiler’s virus (5, 132, 134). These cells compensate for the lack of T-cells during HSV-1 infection and prevent mortality due to encephalitis (5). Recent studies by Nandakumar et al., have shown that NK cells play an important role in limiting encephalitis as shown in in vivo experiments in mice (121). Results from this group suggest that NK cells can compensate for the loss of CD4 T helper cell function. Fatal B virus infection in humans is very similar to HSV-1 encephalitis in humans. Therefore, taking into consideration all the important aspects of NK cells, it is important to study the roles played by NK cells during B virus infection.
1.2 Part II: RESEARCH GOALS

In humans B virus reaches the CNS quickly, often in a matter of days following virus infection resulting in severe neuropathological disease leading to death. Zoonotic infection of B virus is of prime concern for the personnel working with macaques. B virus infection in macaques results in a robust adaptive immune response, however, the same is not true in case of humans. Therefore, it is important to understand how the virus behaves in a foreign host and how it suppresses immune responses required for maintaining a balance between virus replication and immune response. Previous results from our lab suggested that B virus has some evasion mechanisms that result in rendering innate immune responses insufficient, which might ultimately affect the adaptive immune response. Although, NK cells belong to innate immunity, they are capable of producing both Th1 and Th2 cytokines. They can also activate DCs to efficiently present antigen to T-cells. Because NK cells play an important role in forming this bridge between innate and adaptive immunity, it is critical to understand how B virus may impede or subvert the innate immune responses during zoonotic infection. Therefore, we hypothesize that B virus evades host innate immune responses by down regulating signals for activation and migration of natural killer cells in a foreign host. To test this hypothesis, we focused on the regulation of MHC class I, which plays an important role in regulating CD8+ T-cell activity (adaptive immune response) and NK cell activity (innate immune response). Because these responses are responsible for lysing the infected cell, the virus is prevented from spreading to other cells by cell fusion. These studies were designed to identify and understand specific evasion mechanisms employed by B virus that might predictably lead to rapid replication and
spread of virus and suppression of immune response. Basing on the previous research on HSV-1 in other labs, we designed our experiments to test our hypothesis using a cell culture based system that will allow us to examine the factors that determine NK cell activity or inhibition of NK cell activity during B virus infection at a cellular level. To achieve this, we proposed the following specific aims:

1. Identify how regulation of MHC class I in B virus infected HFF cells proceeds.
2. Identify how B virus infection down regulates factors necessary for NK cell activation leading to NK cell failure to respond to B virus infected cells.
2 MATERIALS AND METHODS

2.1 Cells and viruses

Human foreskin fibroblast cells (HFF, ATCC # CRL-2097) obtained from ATCC (Manassas, VA) were maintained as a monolayer in minimal essential media (MEM), purchased from Mediatech, Inc. (Manassas, VA) supplemented with 10% fetal bovine serum (FBS) purchased from Atlanta Biologicals, Norcross, GA, 1% non-essential amino acids, and 1% sodium pyruvate. Passages between 6 and 13 were used for all experiments with HFF cells. Macaque kidney epithelial cells (MK2, ATTC# CCL-7) were obtained from ATCC and were maintained as a monolayer in Dulbecco’s Modified Eagle’s Media (DMEM, Mediatech, Inc.) with 10% FBS. B virus lab strain E2490, passages 71-73, originally from Dr. E. Hull, HSV-1 strain MacIntyre (Lot VR-539) purchased from ATCC, and HVP-2 lab strain X2980 from Southwest Foundation for Biomedical Research, San Antonio, TX were used in the experiments presented. All virus stocks were prepared using Vero cells (Lot # CCL-81) obtained from ATCC. B virus lab strain E2490, HSV-1 strain MacIntyre (Lot # VR-539 from ATCC), and HVP-2 lab strain X2980 were used in our experiments. All experiments with B virus were done in the CDC approved Bio-safety Level-4 (BSL-4) laboratory in compliance with the Biosafety in Microbiological and Biomedical Laboratories manual (BMBL, 5th edition) and the U.S. Department of Justice’s Homeland Security directives on Select Agents. Georgia State University is a registered Select Agent Facility in good standing.
2.2 Virus stocks and plaque assay

Vero cells were grown to sub-confluency in roller bottles after which cells were infected with 0.01 multiplicity of infection (MOI) for 48 hpi (hours post-infection). Infected cells were then collected, centrifuged at 1000 rpm for 10 minutes at room temperature, and the subsequent pellet re-suspended in fresh MEM with 2% FBS. The virus cell lysate was rapidly frozen–thawed three times to disrupt cells for release of virus. Virus infectivity in different cell lines was determined using standard plaque assays in order to quantify the efficiency of plating or the amount of virus needed for infection to achieve equivalent results regardless of the cell line used. For the plaque assay, Vero or HFF cells were grown to sub-confluency in 6-well plates. Media were removed and serially diluted virus 1:10 to a final dilution of $1 \times 10^{10}$ with each dilution added to two wells. Virus was adsorbed in 400 µl for 1 hr at 37°C in a 5% CO$_2$ incubator. At 1 hpi media was removed and fresh MEM media with 1% methylcellulose was added to each well. The plates were incubated for 48 hpi. After 48 hrs media was removed and the wells were washed twice with PBS (Phosphate buffer saline) and fixed with 100% methanol. Fixed cells were stained with crystal violet and the number of plaques was counted. HSV-1 infection in MK2 cells is abortive, however, immediate-early protein expression was observed in independent studies (I.Patrusheva, R.Farah., personal communication).

2.3 Western Blot Analysis

HFF cells were infected with HSV-1 or B virus at a multiplicity of Infection (MOI) 10 to uniformly infect all cells simultaneously to detect total antigen expression of HLA-A, -B, -C, HLA-E, and HLA-G. For detecting the presence of total MHC Class 1 polypeptide-related chain A
MICA), HFF cells were infected with HSV-1 or B virus at a MOI 5. Prepared infected cell lysates were collected and re-suspended in using Laemmili sample buffer containing 5% 2- mercaptoethanol. The solution was boiled for 3 minutes and samples were fractionated on a 10% SDS-PAGE polyacrylamide gel. Subsequently, proteins were transferred onto a nitrocellulose membrane (0.45 µm membrane, GE Healthcare, Piscataway, NJ) by electroblotting for 2 hours. Transferred proteins were probed with mouse anti-human HLA-A, -B, -C clone LY5.1 antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) at a dilution of 1:100 was used to detect total HLA-A, -B, -C antigen content in HFF cells. Mouse anti-human HLA-E clone MEM-E/02 (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200 was used to determine total HLA-E expression in HFF cells. Mouse anti-human HLA-G clone 4H84 (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200 was used to measure total HLA-G protein. For detection of total MICA, mouse anti-human MICA purchased from Abcam was used at a dilution of 1:1000. Rabbit anti-human GAPDH (purchased from Imgenex Corp., CA) was used at a dilution of 1:500 to detect GAPDH expression to evaluate consistency of loading volume of individual wells. Goat anti-mouse antibody conjugated to HRP and goat anti-rabbit antibody conjugated to HRP (Pierce, Rockford, IL) were used at dilutions of 1:20,000 to detect the mouse anti-human and rabbit anti-human primary antibodies, respectively. Respective bands were detected using ECL plus western blotting detection reagent (GE Healthcare).

2.4 Flow Cytometry

HFF and MK2 cells were infected at sub-confluency (MOI 10) or uninfected. At 18 hours post infection (hpi), cells were washed with phosphate buffered saline (PBS). Cells
were trypsinized and fixed with 4% paraformaldehyde at room temperature for 1 hour. Fixed cells were washed three times with 1% Bovine Serum Albumin (BSA) in PBS. Cells were then labeled with mouse monoclonal anti-human HLA class I clone W6/32 conjugated to FITC (Sigma-Aldrich, St. Louis, MO) according to manufacturer instructions (10µl/ 1 million cells), and subsequently incubated at room temperature for 1 hour in darkness. Labeled cells were washed two times in cold PBS and the cells were analyzed using BD FACS Canto (BD Biosciences, San Jose, CA). Protein expression of HLA-A, -B, -C was measured in terms of the mean fluorescence intensity (mfi) emitted by the labeled cells. Uninfected, unlabeled cells were used as negative control to assess auto-fluorescence. Mean fluorescent intensity of labeled cells was normalized by subtracting the mean fluorescent intensity of unlabeled cells from mean fluorescent intensity of labeled cell. Ratio of mean fluorescent intensity was measured using the formula:

\[
\frac{\text{Mean fluorescent intensity of infected cells}}{\text{Mean fluorescent intensity of uninfected cells}}
\]

For detection of MICA/MICB surface expression, HFF cells were infected at a MOI 5, or left uninfected. At 12 and 24 hours post infection (hpi), cells were washed with phosphate buffered saline (PBS). Cells were prepared for flow cytometry as explained before. Cells were labeled with anti-MICA/MICB antibody conjugated to PE (20 µl/ 1 million cells) purchased from Biolegend, San Diego, CA. Uninfected cells that were unlabeled were used as negative control to assess auto-fluorescence. Data analyses were performed using BD FACS Canto (BD Biosciences, San Jose, CA).
2.5 PBMC isolation

Whole blood was drawn from two healthy donors who were sero-negative for B virus and HSV-1 antibodies as determined by western blot. Whole blood (10 ml) was layered onto 4 ml Ficoll-Paque PLUS purchased from GE Amersham (Piscataway, NJ) in 15 ml conical tubes. PBMCs (peripheral blood mononuclear cells) were separated by density gradient by spinning at 2000 rpm at room temperature for 30 minutes with the brake set to off. Three layers are formed, with the top layer containing plasma and platelets and the bottom layer containing red blood cells. A buffy coat, which contains all the PBMCs, was present between the top and bottom layer. The buffy coat was collected and washed in excess PBS to eliminate platelets and plasma by spinning at 800 rpm at room temperature for 10 minutes. The resultant pellet was washed again with RBC lysis buffer (room temperature) to remove red blood cells in the pellet. The pellet containing PBMCs was washed again with PBS twice at room temperature. Supernatant was discarded and the pellet was re-suspended in excess cold RPMI with 10% FBS (25 ml media for every 100 ml whole blood), then stored at 4ºC overnight for subsequent PBMC assays and NK cell isolation.

2.6 NK cell isolation

Natural killer cells (NK cells) were isolated using MACS NK cell isolation kit on PBMCs (described above) obtained from laboratory donors lacking antibodies against the specific viruses described (Miltenyi Biotec, Auburn, CA). The NK cells were separated using indirect magnetic labeling, in which NK cells were obtained by negative selection. This resulted in isolation
of nearly pure populations of NK cells that remained un-activated by the process. About 5% NK cells were isolated from donor PBMCs.

2.7 Cytokine/ chemokine production in infected HFF cells

HFF cells were infected in a minimum volume containing B virus or HSV-1 at a MOI 5, or mock infected using vero cell lysates and incubated in 37°C, 5% CO₂ incubator for 1 hour to facilitate adsorption. At 5 and 23 hours post-adsorption (6 and 24 hours post infection, respectively), supernatants from the different wells were collected to quantify chemokines and cytokines released by infected cells for NK cell migration and activation. Cytokines and chemokines were detected using Liquichip™ assay and instrumentation from Qiagen, Valencia, CA.

2.8 PBMC assay

HFF cells were infected with B virus, HSV-1, or mock infected with uninfected Vero cell lysates. At 6 hours post-infection (hpi), PBMCs isolated (as described above) from antibody-negative donors were added to each well at 1:50 target to effector ratio. The infected cells overlaid with PBMCs were incubated for 18 hrs and the supernatants were then collected to measure the cytokines and chemokines produced using Liquichip™ assay. The presence of perforin and granzyme B (indicative of activated NK cell killing) in the supernatants was detected using an ELISA. Centrifuged pellets prepared from cell lysates were solubilized in Laemmilli sample buffer containing with 5% 2- mercaptoethanol. These samples were fractionated on a 10% acrylamide gel and the proteins were transferred to nitrocellulose membrane by electroblotting. Transferred proteins were reacted with Anti-phospho-syk and syk protein at dilutions
of 1:1000 and 1:5000 respectively to identify involvement of syk dependent activation receptors expressed by cells from the PBMC population.

2.9 NK cell activation assay

To measure NK cell activation, HFF cells were infected with B virus, HSV-1, or uninfected cell lysates. At 6 hours post-infection (hpi), NK cells isolated (as described above) from antibody-negative donors were added to each well at 1:5 target to effector ratio. The infected cells overlaid with NK cells were incubated with or without the media containing IL-2 (70 U/ml) for 18 hrs. The presence of Th1 and Th2 cytokines produced upon NK cell exposure to infected HFF cells was quantified using LiquiChip™ technology. The presence of perforin and granzyme B in the supernatants was detected using ELISA. In order to determine if NK cell activity is syk dependent the same protocol described above for PBMC’s was used for NK cell assays.

2.10 Liquichip assay

A Liquichip™ assay was performed using Luminex (xMAP technology) from Qiagen, Valencia, CA. The assay was performed as per the manufacturer’s instructions. Liquichip™ human cytokine detection kit was purchased from Millipore, Billerica, MA. The samples and controls were mixed with beads coated with specific antibody so that each specific bead can capture a specific analyte (cytokine) from the sample. The bead sample mixture was then incubated with a biotinylated detection antibody. This reaction mixture was incubated with streptavidin-PE
conjugate, sorted and interrogated by a laser which quantified each binding to each specifically coated microbead population. Data analysis was performed using Milliplex™ Analyst software.

2.11 Perforin and Granzyme B release assay

Supernatants from NK cells with or without treatment with IL-2, as well as PBMC co-cultured cell supernatants were used to quantify released perforin and Granzyme B, which were indicative of NK cell cytolytic activity. Perforin and Granzyme B were measured using a perforin ELISA kit and Granzyme B ELISA kit purchased from Abcam, Cambridge, MA. These assays were sandwich ELISAs and were performed according to manufacturer’s instructions. The wells of the microtiter plates were coated with anti-perforin or anti-granzyme B antibody and incubated with respective samples. After one-hour incubation, wells were washed three times with washing buffer. Each well is then incubated with biotinylated anti-perforin or granzyme B. After the incubation, the wells were washed again three times with the washing buffer. The antibody-antigen-antibody interaction was detected using streptavidin-HRP. Tetramethylbenzidine (TMB) substrate was used for the development of color in the wells with supernatants that have detectable amounts of perforin or granzyme B. The optical density (OD) values were converted to pico grams/ milliliter (pg/ml) using a standard curve.

2.12 Statistical Analysis

Data obtained by FACS for HLA-A, -B, -C expression were repeated four times and standard deviation was calculated. We used paired, two-tailed Students t-test to calculate the p-values between two different samples. A significant difference was considered between unin-
fected and B virus or uninfected and HSV-1 infected cells or HSV-1 and B virus infected cells if the p-values were found to be ≤ 0.05.

Data dealing with cytokines, chemokines, granzyme B, and perforin measurements were analyzed using one-way analysis of variance (one-way ANOVA) to compare mock infected, HSV-1, and B virus infected samples. One-way ANOVA, however, gives an overall difference between different samples. For that reason, a multiple pair-wise comparison test, such as Student Newman-Keuls test (SNK test) was used as a post-hoc ANOVA. If q-values obtained from SNK test were found to be more than the critical value for α₁=0.05, then the two samples were considered to be significantly different from each other.
3 RESULTS

3.1 Part I: MHC class I regulation in B virus infected cells.

Down-regulation of surface expression of MHC class I is a hallmark of all herpes viruses. HSV-1 and HSV-2 US12 encodes for ICP47, which binds to host TAP and prevents peptide loading onto MHC class I. This block leads to sequestration of MHC class I in the ER and subsequent down-regulation of surface MHC class I. B virus, a non-human simplex virus, very similar to HSV-1 and 2 contains US12 gene encoding ICP47. B virus ICP47 does not have TAP binding domain required for down regulating TAP. The absence of TAP binding domain in ICP47 protein is also observed in other non-human primate simplex viruses such as HVP-2 and SA8. Therefore, we hypothesize that B virus regulation of MHC class I is different from HSV in natural and foreign hosts. The following experiments were conducted to test this hypothesis.

Virus infectivity. In order to determine the efficiency of B virus infection in HFF cells (cells derived from a foreign host or one in which the virus had not co-adapted to), virus infectivity in Vero cells was compared with virus infectivity in HFF cells using plaque assay. Similarly, HSV-1 and HVP-2 virus infectivity was also determined in Vero cells and HFF cells. In contrast, MK2 cells are derived from a natural host, macaque monkeys, and serve as such for B virus infection while these serve as a foreign host cell line for HSV-1 infection. Therefore, virus infectivity of HSV-1 and B virus was also determined in MK2 cells. The results indicated that B virus infectivity in HFF cells was ~ 3 times less efficient than virus infectivity in Vero cells derived from
the African green monkey. As a result of these findings, three times more B virus was required to infect HFF cells compared to that required for Vero cells to obtain equivalent plaque forming units (PFU). Results for HSV-1 indicated that there was no difference in efficiency of virus infectivity in Vero cells or HFF cells. Interestingly, 10 times more B virus was required to infect MK2 at equivalent levels to that in Vero cells. HSV-1 infection in MK2 cells was abortive with only IE (immediate early) and E (early) protein expression detected in independent studies (I.Patrusheva and R.Farah, personal communication). HVP-2, a virus naturally found in baboons was 10 times less efficient replicating in HFF cells when compared to replication in Vero cells (Table 1).
Table 1: Virus infectivity in different cell lines compared to Vero cells. Virus infectivity in different cell lines was quantified by plaque assay.

<table>
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<tr>
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<th>HFF</th>
<th>MK2</th>
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<tr>
<td><strong>B Virus</strong></td>
<td>3X</td>
<td>10X</td>
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<tr>
<td><strong>HSV-1</strong></td>
<td>1X</td>
<td>Abortive</td>
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<tr>
<td><strong>HVP-2</strong></td>
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* ND: Not determined
**Total antigen expression of HLA-A, -B, -C in B virus infected HFF cells.** Once equivalent virus loads were determined for each cell line, experiments proceeded to evaluate the effect of each virus in each cell system with respect to virus:host cell interactions. HLA-A, -B, and -C are predominantly antigen presenting MHC 1a molecules. It is well known that HSV-1 down regulates MHC Class 1 at a post-translational level by sequestering MHC class I molecules in the endoplasmic reticulum (68, 184). This sequestration results in down-regulation of surface MHC class I expression. Western blot analysis of MHC Class 1 total protein in HSV-1 infected cells, however, showed no reduction in levels. To determine if B virus regulates MHC class I at translational level, we examined the effect of B virus infection on total antigen expression of HLA-A, -B, -C in HFF cells. Cells were infected with B virus or HSV-1 at 10 MOI, and for controls, cells were either left uninfected or mock infected with uninfected cell lysates. At 6 and 18 hours post infection (hpi), cells were lysed using Laemmli sample buffer containing with 5% 2-Mercaptoethanol. Following fractionation and western blotting, the presence of HLA-A, -B, -C in these samples was measured. Data indicated similar levels of HLA-A, -B, -C in uninfected, mock, B virus infected and HSV-1 infected HFF cells at 6 and 18 hpi. These data suggest that B virus does not interfere with HLA-A, -B, -C expression at a translational level (Figure 5).
Figure 5: Total HLA-A, -B, -C antigen expression in B virus or HSV-1 infected HFF cells. There was no significant difference in the total MHC class I protein expression between infected and uninfected cells at 6 and 18 hpi.
**Regulation of MHC class I surface expression in B virus infected cells.** Most herpes viruses are known to down regulate cell surface MHC class I. From the western blot results, it was apparent that MHC Class 1 gene expression was not down regulated in either. B virus, or the closely related HSV-1 infected HFF cells. The next logical question posed as a result of these data was whether reduced levels of cell surface in HLA-A, -B, -C is observed in B virus infected HFF cells. To address this question, experiments were designed to measure HLA-A, -B, -C surface expression on B virus infected cells using flow cytometry at 18 hpi. As a control, HSV-1 infected cells were used, since this virus is known to down-regulate HLA-A, -B, -C surface expression. Results of the experiments show that HSV-1 infected HFF cells showed ~65% HLA-A, -B, -C down-regulation when compared to uninfected HFF cells. On the other hand, B virus infected HFF cells showed only 32% reduction compared to uninfected cells (Figure 6A and 6B). Together, these results suggest that B virus does not down-regulate HLA-A, -B, -C surface expression as efficiently as HSV-1 in HFF infected cells ($p < 0.05$), although there is significant decrease compared to uninfected cells ($p \leq 0.05$).
Figure 6A: FACS analysis showing surface expression of HLA-A, -B, -C on B virus or HSV-1 infected HFF cells at 18 hpi. Down-regulation of HLA-A, -B, -C on the cell surface is greater in HSV-1 infected HFF cells as compared to B virus infected HFF cells at 18 hpi. This figure is a representative of four independent experiments.
Figure 6B: Ratio of mean FITC intensities of infected/uninfected. Mean FITC values were calculated from four independent experiments. Mean FITC of uninfected HFF cells labeled with HLA-A, -B, -C was given an arbitrary value of 1 and mean intensities of infected HFF cells calculated relative to uninfected HFFcells. The error bars represent standard deviation obtained from the ratio of mean FITC intensities. \( p\)-values were measured using Student t-test.
Regulation of HLA-A, -B, -C cell surface expression in B virus infected cells derived from natural vs. foreign hosts. The previous data show that B virus does not down regulate HLA-A, -B, -C cell surface expression efficiently in HFF cells relative to HSV-1. To determine if this observation was consistent in cells derived from macaques (MK2), the natural host for B virus, we infected MK2 cells with B virus or HSV-1 (MOI 10) as described in Materials and Methods. We analyzed the infected cells at 18 hpi for HLA-A, -B, -C surface expression using flow cytometry, since at that point turnover of surface MHC Class 1 molecules occurs. Data showed that B virus did not down-regulate HLA-A, -B, -C surface expression as efficiently as HSV-1 in macaque host cells, in spite of the observations that HSV-1 underwent an abortive infection in these cells. B virus infected cells showed ~24% reduction of HLA-A, -B, -C as compared to uninfected cells, while HSV-1 infected cells showed 59% down-regulation compared to uninfected cells (Figure 7A and 7B). The p-value for HSV-1 infected cells is < 0.05, indicating a significant reduction in cell surface expression of HLA-A, -B, and -C between HSV-1 infected and uninfected cells. The p-value for B virus infected cells, however, is ≥ 0.05 indicating that there is no difference in MHC class I expression between uninfected and B virus infected MK2 cells. These data suggest that B virus does not block normal levels of surface MHC Class 1 molecules in cells derived from macaque hosts, nor does it interfere in cells derived from human hosts. As a result, it is likely that B virus infected cells are susceptible to cytotoxic lymphocytes during the course of infection.
Figure 7A: FACS analysis of HLA-A, -B, -C expression on B virus and HSV-1 infected MK2 cells at 18 hpi. There was no difference in the HLA-A, -B, -C surface expression in B virus or HSV-1 infected HFF and MK2 cells.
**Figure 7B: Ratio of mean FITC intensities of infected/uninfected.** Mean FITC values were calculated from three independent experiments. Mean FITC values and *p*-values were calculated in a similar manner as explained in figure 6B. The error bars represent standard deviation obtained from the ratio of mean FITC intensities.
Effects of Viral Proteins regulating protein loading of the MHC Class 1 molecules. HSV-1 and HSV-2 encode for a protein, ICP47 containing a TAP binding domain responsible for sequestering HLA-A, -B, -C molecules in the ER. Non-human primate simplex viruses such as B virus, Herpesvirus Papio-2 (HVP-2, Cercopitheine Herpesvirus 16), and Simian Herpesvirus (SA8) encode for an ICP47 protein that does not have a TAP binding domain similar to HSV-1 and HSV-2. We wanted to look at HLA-A, -B, -C regulation in a similar virus that lacks TAP binding domain. For this reason, we chose HVP-2, which is very close to B virus. This would allow us to predict if lack of TAP binding domain in ICP47 protein plays a role in inefficient down-regulation of HLA-A, -B, -C. HFF cells were infected with HVP-2 or HSV-1 and HLA-A, -B, -C surface expression was analyzed using flow cytometry. Our results showed that HVP-2 behaves in a similar way to B virus in down regulating HLA-A, -B, -C surface expression on infected cells (Figure 8A and 8B). These results do not indicate direct correlation between the lack of TAP binding domain and inefficient HLA-A, -B, -C down-regulation, however, these results do indicate that non-human primate simplex viruses might regulate HLA-A, -B, -C expression differently from human simplex viruses.
**Figure 8A: FACS analysis of HLA-A, -B, -C surface expression in HVP-2 or HSV-1 infected HFF cells.** HVP-2 is closely related to B virus and also lacks TAP binding domain in its ICP47 protein similar to B virus. HVP-2 down-regulation of HLA-A, -B, -C was significantly less than HSV-1 down-regulation of HLA-A, -B, -C.
Figure 8B: Ratio of mean FITC intensities of infected/uninfected. Mean FITC values were calculated from three independent experiments. Mean FITC values and $p$-values were calculated in a similar manner as explained in figure 6B. The error bars represent standard deviation obtained from the ratio of mean FITC intensities.
HLA-E expression correlates with HLA-A, -B, -C expression in B virus or HSV-1 infected cells. HLA-E cell surface expression is dependent on HLA-A, -B, -C cell surface expression and stability, because HLA-E presents HLA-A, -B, -C leader peptide via TAP. Our data suggested 32% down-regulation of HLA-A, -B, -C in B virus infected cells and 65% down-regulation in HSV-1 infected cells. Therefore, we analyzed HLA-E expression in B virus or HSV-1 infected cells using western blot analysis. At 6 hpi, data revealed reduced expression of HLA-E in B virus infected HFF cells. A significant reduction in HLA-E expression is observed in HSV-1 infected cells compared to uninfected cells. At 18 hpi, there is total reduction in HLA-E expression in virus infected cells compared to the controls (Figure 9). These data suggest that HLA-E expression correlates with HLA- A, -B, -C expression in B virus and HSV-1 infected cells.
Figure 9. Western blot analysis of HLA-E expression in B virus or HSV-1 infected HFF cells at 6 and 18 hpi. HLA-E expression correlates HLA-A, -B, -C expression in HSV-1 or B virus infected HFF cells. HLA-E was reduced by 6 hpi more prominently in HSV-1 infected cells as compared to B virus infected cells.
**Differential expression of HLA-G in B virus infected and HSV-1 infected cells.** HLA-G is not expressed in all cells and is mostly observed on placenta protecting the fetus from maternal NK cell activity. The regulation of HLA-G expression in HSV-1 infected HFF cells has not been previously described. Total HLA-G expression in B virus infected, HSV-1, and uninfected HFF cells was analyzed using western blot analysis. These data show that HSV-1 infected cells at 6 hpi expressed proteins corresponding to 21, 34, and 37 KDa when western blots were probed with anti-HLA-G antibody. These bands correspond to each different isoform of HLA-G. B virus infected HFF cells showed little expression of HLA-G (34 KDa isoform) while uninfected cells showed no expression of any of the HLA-G isoforms. By 18 hpi, only faint band was noted corresponding to 34 KDa in case of HSV-1 infected HFF cells with no HLA-G observed in B virus and uninfected cells (Figure 10). Thus, HSV-1 and B virus appear to differentially regulate HLA-G expression in infected HFF cells.
Figure 10: Western blot analysis of HLA-G expression in HSV-1 or B virus infected HFF cells at 6 and 18 hpi. Differential expression of HLA-G is observed in HSV-1 and B virus infected HFF cells. Here, different isoforms of HLA-G was observed in HSV-1 infection, however, only one isoform of HLA-G was observed in B virus infected HFF cells, which disappears by 18 hpi.
3.2 PART II: Natural Killer cell regulation during B virus infection in HFF cells.

Although HSV specific CTLs are generated by antigen presenting cells, these CTLs fail to recognize HSV-1 infected cell due to lack of antigen presenting MHC class I on the cell surface. The CTLs that are generated are important in limiting the spread of virus and in inhibiting reactivation. Because of MHC class I down-regulation on HSV infected cells, these cells are susceptible to NK cell lysis. NK cell recognition limits the virus spread and replication during primary infection. Thus, a balance between HSV infection and immune response is maintained that helps HSV to co-exist in a natural host. During fatal zoonotic B virus infection, this balance might not be maintained. Strong innate immune responses are important during primary infection and previous studies in our lab have shown that some of the innate immune responses are impaired during B virus infection of human cells. One of the important components of innate immunity is NK cell activity. From our results it is evident that B virus does not down regulate MHC class I, therefore we hypothesize that B virus infected cells are resistant to NK cell lysis. Consequently, we tested for different factors that effect NK cell activity during B virus infection in the next series of experiments.

**MICA/MICB expression in B virus infected HFF cells.** MICA and MICB belong to the family of MHC class I, however, they bind to NKG2D, activation receptor of NK cells. The other family members that bind to NKG2D are ULBP1-4. Therefore, MICA, MICB, and ULBP proteins are involved in the activation of NK cells while MHC class I proteins are involved in the inhibition of NK cells. The NKG2D activation ligands can overcome the inhibitory effect of MHC class I. Therefore, in this experiment we measured the expression of MICA/MICB in B virus and HSV-1
infected HFF cells at 12 and 24 hpi. HFFs do not express ULBP proteins under normal conditions, however MICA/MICB are expressed at a basal level. Here we examined the total antigen expression of MICA in infected cells at 12 and 24 hpi using western blot analysis. HFF cells were infected with HSV-1 or B virus at 5 MOI or remained uninfected for 12 and 24 hpi. Western blot analysis shows reduced expression of MICA in B virus and HSV-1 infected HFF cells by 24 hpi (Figure 11A). We then measured the surface expression of MICA/MICB using FACS analysis, as we did not test for the total antigen expression of MICB in infected cells because of the lack of commercially available antibody against MICB. MICA/MICB antibody detects the presence of both MICA and MICB proteins on the cell surface. Our results showed an up-regulation of MICA/MICB on the cell surface of infected cells at 12 and 24 hpi, thus indicating that the up-regulation is because of MICB rather than MICA (Figure 11B).
Figure 11A: Western blot analysis of total antigen expression of MICA in B virus and HSV-1 infected HFF cells at 12 and 24 hpi. There is no difference in MICA expression between uninfected and infected cells at 12 hpi. By 24 hpi, B virus and HSV-1 down regulate MICA as compared to uninfected HFF cells.
Figure 11B: FACS analysis of MICA/MICB surface expression in B virus and HSV-1 infected cells at 12 and 24 hpi. An up-regulation of MICA/MICB is observed at 12 and 24 hpi in infected cells, most probably contributed by MICB. This increase in surface expression could be a stress response rather than virus specific.
Expression of cytokines and chemokines involved in NK cell migration and activation in B virus and HSV-1 infected HFF cells. During infection, a cell will produce certain chemoattractants for the migration of NK cells and cytokines to prime them. Therefore, NK cells need both activation ligands and cytokines that can help in activation ligand-receptor binding in order for their activation. IFN-α and IL-15 are cytokines implicated in playing a role in activating NK cells. Chemokines that are involved in NK cell migration and activation are IL-8, IP-10, MIP-1a, MIP-1b, and MCP-1. Therefore, in our experiment we quantified the expression of above cytokines/chemokines produced by mock, HSV-1, or B virus infected HFF cells at 6 and 24 hpi. B virus or HSV-1 infected HFF cells do not induce the expression of any of the cytokines or chemokines described above. In fact HSV-1 and B virus infection of HFF cells leads to down-regulation of INF-α, IP-10, IL-8, and MCP-1 by 24 hpi (Figure 12A-12D respectively). Certain chemokines like MIP-1a and MIP-1b are not induced in mock, HSV-1, or B virus infected HFF cells at 6 and 24 hpi. These combined results suggest that both B virus and HSV-1 do not induce cytokines and chemokines for NK cell migration and activation upon infecting HFF cells (Table 2).
Figure 12A: IFN-α expression by mock, B virus, or HSV-1 infected HFF cells at 6 and 24 hpi.
One-way ANOVA of mock, HSV-1, and B virus infected samples at 24 hpi yields a p-value of 0.018. Multiple comparisons between the samples using SNK test shows a significant difference between mock and HSV-1 or mock and B virus infected cells with q values greater than the critical values at 24 hpi. The error bars represent standard errors.
Figure 12B: Liquichip analysis of IP-10 expression in mock, B virus or HSV-1 infected at 6 and 24 hpi. One-way ANOVA of mock, HSV-1, and B virus infected samples at 24 hpi yields a p-value of 0.03. Multiple comparisons between the samples using SNK test shows a significant difference between mock and B virus infected cells with q values greater than the critical values. However, there is no difference between mock and HSV-1 treated samples and that is because of the high variance in the mock treated samples. The error bars represent standard errors.
Figure 12C: Detection of expression of IL-8 at 6 and 24 hpi by mock, B virus, or HSV-1 infected cells at 6 and 24 hpi using liquichip analysis. One-way ANOVA of mock, HSV-1, and B virus infected samples at 6 and 24 hpi yields a p-value of <0.0001 for both time points respectively. Multiple comparisons between the samples using SNK test shows a significant difference between mock and HSV-1 or mock and B virus infected cells with q values greater than the critical values. The error bars represent standard errors.
*Mock-infected HFF cells induced > 6000 pg/ml, higher than the detection levels, but plotted with the value for maximum detection level.

**Figure 12D: MCP-1 expression in B virus and HSV-1 infected cells at 6 and 24 hpi using liquichip assay.** One-way ANOVA of mock, HSV-1, and B virus infected samples at 6 and 24 hpi yields a p-value of <0.0001 and 0.00048 respectively. Multiple comparisons between the samples using SNK test shows a significant difference between mock and HSV-1 or B virus infected cells with q values greater than the critical values. The error bars represent standard errors.
Table 2: Summary of expression of cytokines and chemokines responsible for NK cell migration and activation by mock, HSV-1, or B virus infected HFF cells at 24 hpi

<table>
<thead>
<tr>
<th></th>
<th>Mock Infected HFF</th>
<th>HSV-1 infected HFF cells</th>
<th>B virus infected HFF cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>✔️ (30.27 pg/ml)</td>
<td>⇓ (10.59 pg/ml)</td>
<td>✗ (6.71 pg/ml)</td>
</tr>
<tr>
<td>IL-15</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>IP-10</td>
<td>✔️ (21 pg/ml)</td>
<td>✗ (4 pg/ml)</td>
<td>✗ (5 pg/ml)</td>
</tr>
<tr>
<td>IL-8</td>
<td>✔️ (2539 pg/ml)</td>
<td>⇓ (43.75 pg/ml)</td>
<td>⇓ (22.79 pg/ml)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>✔️ (5291 pg/ml)</td>
<td>⇓ (2364 pg/ml)</td>
<td>⇓ (239 pg/ml)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
</tbody>
</table>
Cytokines/chemokines released by PBMC upon exposure to infected HFF cells. From our results we observed that B virus as well as HSV-1 infected HFF cells do not induce cytokines and chemokines involved in NK cell migration and activation. Resident tissue NK cells, however, can mobilize and interact with infected target cells. For influx of additional NK cells during an infection, other immune cells in circulation can be regulated to induce immune responses against the infected cells. Therefore, we tested for cytokines and chemokines involved in migration and activation of NK cells by immune cells in circulation upon exposure to infected cells. For this purpose, we isolated PBMCs from sero-negative individuals. By using PBMCs from sero-negative individuals we are eliminating the possible reaction by adaptive immune responses (T-cell and B cell activity) for HSV-1 specific antigen and at the same time determine the innate immune responses induced during primary infection by cells in circulation. Also, these experiments reflect a primary infection where all cells are exposed to infection for the first time. In our experiments, we infected cells with mock cell lysate, HSV-1, or B virus at MOI of 5. At 6 hpi, PBMCs were added to the infected cells (overlay) and co-cultured for 18 hrs. The supernatants were collected to test for cytokines and chemokines mentioned in the above experiment. Our results show that IFN-α and IP-10 are up-regulated when PBMCs were sub-cultured with HSV-1 infected HFF cells. The same is not observed in case of B virus infection, therefore, we conclude that B virus does not induce responses for activation of NK cells either by infected cells or by regulating circulatory effector cells (PBMCs) (Figure 13A to Figure 13E, Table 3).
Figure 13A: IFN-α expression during PBMC exposure to mock, HSV-1, B virus infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of <0.0001. Multiple comparisons between the samples using SNK test shows a significant difference between HSV-1 and mock or B virus. The error bars represent standard errors.
Figure 13B: IP-10 expression during PBMC exposure of mock, HSV-1, or B virus infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.038. Multiple comparisons between the samples using SNK test shows a significant difference between HSV-1 and mock. B virus samples had values with high variance from the mean and therefore did not show any significant difference as compared to mock or HSV-1. The error bars represent standard errors.
Figure 13C: IL-8 expression during PBMC exposure of mock, HSV-1, or B virus infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of <0.0001. Multiple comparisons between the samples using SNK test shows a significant difference between mock and the infected cell supernatants. The error bars represent standard errors.
Figure 13D: MCP-1 expression during PBMC exposure of mock, HSV-1, or B virus infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.132. Thus, there is no difference between mock and infected cells. The error bars represent standard errors.
Figure 13E: MIP-1β expression during PBMC exposure of mock, HSV-1, or B virus infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.421. Thus, there is no difference between mock and infected cells. The error bars represent standard errors.
Table 3: Summary of expression levels of cytokines and chemokines responsible for NK cell migration and activity during B virus and HSV-1 infection upon exposure to PBMCs.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mock Infected HFF</th>
<th>HSV-1 infected HFF cells</th>
<th>B virus infected HFF cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-α</td>
<td>✗ (0.09 pg/ml)</td>
<td>† (282.6 pg/ml)</td>
<td>† ? (36.5 pg/ml)</td>
</tr>
<tr>
<td>IL-15</td>
<td>✗ (&lt;= 10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>IP-10</td>
<td>✔ (6191.5 pg/ml)</td>
<td>† (12300 pg/ml)</td>
<td>No Difference</td>
</tr>
<tr>
<td>IL-8</td>
<td>✔ (5544 pg/ml)</td>
<td>‡ (1780 pg/ml)</td>
<td>‡ (478 pg/ml)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>✗ (&lt;= 10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (Undetectable)</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>No Difference</td>
<td>No Difference</td>
<td>No Difference</td>
</tr>
</tbody>
</table>
Th1 and Th2 cytokines induced by NK cells upon exposure to B virus or HSV-1 infected HFF cells. So far we have determined a number of specific factors that can induce NK cell activity or inhibition, however, not all aspects were tested. To determine if primary NK cells from sero-negative donors are activated upon exposure to B virus infected cells, supernatants were tested for Th1 and Th2 cytokines that are induced by activated NK cells. Expression of Th1 cytokines, TNF-α, IFN-γ, GM-CSF, and IL-2, was measured in the supernatants using LiquiChip™ assay (Figure 14A to Figure 14D, Table 4, 5). Supernatants were also tested for Th2 cytokines, IL-4, IL-5, IL-10, IL-12, and IL-15 (Table 4, 5). In this experiment, NK cells were also treated with IL-2 to see if there is enhanced NK cell activity specific to infection. TNF-α was found to be up-regulated when NK cells were exposed to B virus infection when compared to mock. A significant TNF-α up-regulation was also observed in case of HSV-1 infection. No other Th1 or Th2 cytokines were induced in either case of infection. NK cells treated with IL-2 did not behave any differently, with up-regulation of TNF-α observed in case of NK cells exposed to infected cells compared to mock infected cells. This up-regulation was not IL-2 specific, thus, IL-2 treatment alone is not important for activation of NK cells or for the expression of TNF-α. Therefore, we conclude that TNF-α is induced by primary NK cell upon exposure to B virus infection. The amount of TNF-α production during B virus or HSV-1 infection is less than what is found in physiological concentration, therefore, the expression of TNF-α could be a result of non-specific expression of TNF-α by naïve NK cells (this remains to be tested).
Figure 14A: GM-CSF expression in NK cells exposed to mock, HSV-1, or B virus infected HFF cells. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.001. Multiple comparisons between the samples using SNK test shows a significant difference between mock and the infected cell supernatants. The error bars represent standard errors.
Figure 14B: GM-CSF expression in NK cells with IL-2 treatment exposed to mock, HSV-1, or B virus infected HFF cells. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.00004. Multiple comparisons between the samples using SNK test shows a significant difference between mock and the infected cell supernatants. The error bars represent standard errors.
Figure 14C: TNF-α expression in NK cells exposed to mock, HSV-1, or B virus infected HFF cells. Increased expression of TNF-α is observed in case of B virus infection. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.000134. Multiple comparisons between the samples using SNK test shows a significant difference between B virus and mock and also between HSV-1 and mock. The error bars represent standard errors.
Figure 14D: TNF-α expression in NK cells with IL-2 treatment exposed to mock, HSV-1, or B virus infected HFF cells. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.024. Multiple comparisons between the samples using SNK test shows a significant difference between B virus and mock and also between HSV-1 and mock. The error bars represent standard errors.
Table 4: Summary of cytokines released by NK cells upon exposure to mock, B virus or HSV-1 infected HFF cells for 18 hrs.

<table>
<thead>
<tr>
<th></th>
<th>Mock Infected HFF</th>
<th>HSV-1 infected HFF cells</th>
<th>B virus infected HFF cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF (Th1)</td>
<td>✔ (16.12 pg/ml)</td>
<td>✘ (&lt;10 pg/ml)</td>
<td>✘ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>TNF-α (Th1)</td>
<td>✘ (&lt;10 pg/ml)</td>
<td>↑ (13.29 pg/ml)</td>
<td>↑ (18.03 pg/ml)</td>
</tr>
<tr>
<td>IFN-γ (Th1)</td>
<td>✘ (&lt;10 pg/ml)</td>
<td>✘ (&lt;10 pg/ml)</td>
<td>✘ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>IL-2</td>
<td>✘ (Undetectable)</td>
<td>✘ (Undetectable)</td>
<td>✘ (Undetectable)</td>
</tr>
<tr>
<td>Th2</td>
<td>✘ (&lt;= 10 pg/ml)</td>
<td>✘ (&lt;10 pg/ml)</td>
<td>✘ (Undetectable)</td>
</tr>
</tbody>
</table>
Table 5: Summary of cytokines released by NK cells with IL-2 treatment upon exposure to mock, B virus or HSV-1 infected HFF cells for 18 hrs.

<table>
<thead>
<tr>
<th></th>
<th>Mock Infected HFF</th>
<th>HSV-1 infected HFF cells</th>
<th>B virus infected HFF cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF (Th1)</td>
<td>✔ (124.98 pg/ml)</td>
<td>‡ (10.6 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>TNF-α (T12)</td>
<td>✔ (17.73 pg/ml)</td>
<td>✔ (19.87 pg/ml)</td>
<td>✔ (22.69 pg/ml)</td>
</tr>
<tr>
<td>IFN-γ (Th1)</td>
<td>✔ (27.55 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>IL-2</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Th2</td>
<td>✗ (&lt;= 10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (Undetectable)</td>
</tr>
</tbody>
</table>

* NR: Not relevant
**Th1/Th2 cytokines induced during PBMC exposure to infected HFF cells.** In our previous results we observed that NK cell activating IFN-α and IL-10 were induced in the presence of PBMCs. Because PBMCs also contain NK cells we wanted to test for Th1 and Th2 cytokine expression in the supernatants of infected HFF cells sub-cultured with PBMCs. Although Th1 and Th2 cytokines are also induced by activated T-cells, we can rule that possibility out because these PBMCs were derived from sero-negative individuals that do not have activated T-cells. Therefore, whatever Th1 and Th2 cytokine expression is observed it is presumably induced by NK cells that do not need prior activation. Here, we observe that a significant up-regulation in TNF-α was in case of PBMCs sub-cultured with HSV-1 infected cells, but not in case of B virus infection suggesting that TNF-α could be induced by other cells during HSV-1 infection but only by NK cells during B virus infection (Figure 15C). Significant down-regulation of GM-CSF and IL-2 (Figures 15A and 15B respectively) was observed in infected cells and no expression of IFN-γ was observed in any of the supernatants tested (Table 6). No significant expression of Th2 cytokines was observed (Table 6). These results suggest that NK cells during HSV-1 or B virus infection do not play a role in inducing Th1 or Th2 cytokines.
Figure 15A: Expression of GM-CSF by PBMCs exposed to mock, HSV-1 or BV infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.002. Multiple comparisons between the samples using SNK test shows a significant difference between mock and infected cell supernatants. The error bars represent standard errors.
Figure 15B: Expression of IL-2 by PBMCs exposed to mock, HSV-1 or BV infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of <0.0001. Multiple comparisons between the samples using SNK test shows a significant difference between mock and infected cell supernatants. The error bars represent standard errors.
Figure 15C: TNF-α expression by PBMCs exposed to mock, HSV-1, B virus infected HFF cells for 18 hrs. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.009. Multiple comparisons between the samples using SNK test shows a significant difference between HSV-1 and mock treated cells and HSV-1 and B virus treated cells. The error bars represent standard errors.
Table 6: Summary of cytokines released by NK cells under the influence of PBMCs.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mock Infected HFF</th>
<th>HSV-1 infected HFF cells</th>
<th>B virus infected HFF cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF (Th1)</td>
<td>✔️ (167.08 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>TNF-α (T12)</td>
<td>✔️ (20.52 pg/ml)</td>
<td>🔻 (62.37 pg/ml)</td>
<td>✔️ (30.26 pg/ml)</td>
</tr>
<tr>
<td>IFN-γ (Th1)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>IL-2</td>
<td>🔻 (68 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
<td>✗ (&lt;10 pg/ml)</td>
</tr>
<tr>
<td>Th2</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>
**NK cell expression of cytotoxic granules, perforin and granzyme B, during B virus infection.** Upon activation NK cells can induce either cytokines and/or cause cytotoxicity. NK cells exert cytotoxicity by the production of perforin and granzyme B. Therefore, in this experiment we wanted to investigate whether NK cells engaged early cytolytic activity during B virus or HSV-1 infection. Our results show that neither perforin nor granzyme B is induced during B virus or HSV-1 infection by NK cells (Figure 16A and Figure 16B respectively) with or without IL-2 treatment. IL-2, among other factors, plays an important role in NK cell cytotoxicity, therefore, we see an up-regulation of perforin in mock cells with NK/IL-2 treatment. This up-regulation does not imply cytolysis, but simply the formation of cytotoxic granules naturally occurring in NK cells. Interestingly, up-regulation was not observed in case of HSV-1 or B virus infected cells. From these results we concluded that primary NK cells with or without IL-2 treatment produced TNF-α, but not other cytokines or even cytotoxic granules during B virus infection or HSV-1 infection.
Figure 16A: Release of cytotoxic granule, perforin by NK cells with or without IL-2 treatment during mock, HSV-1, or B virus infection. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.025 and <0.001 for cells exposed to NK or NK/IL-2 respectively. Multiple comparisons between the samples using SNK test shows a significant difference between mock and infected cells exposed to NK or NK/IL-2. The error bars represent standard errors.
Figure 16B: Release of cytotoxic granule, granzyme B by NK cells with or without IL-2 treatment during mock, HSV-1, or B virus infection. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.0003 and 0.008 for cells exposed to NK or NK/IL-2 respectively. Multiple comparisons between the samples using SNK test shows a significant difference between mock and infected cells exposed to NK or NK/IL-2. The error bars represent standard errors.
**Cytotoxic granules release during PBMC exposure to B virus infected HFF cells.** Peforin and Granzyme B are mostly released by activated NK cells or activated CTLs. Because in our experiment we used antibody negative donor PBMCs, we assume that CTLs are not active. Therefore, the perforin or granzyme B if released should most probably be because of NK cells that have been activated either by contact with the infected target cells or under the influence of other effector cells in the PBMCs. Our experimental results show that there is no significant increase in the release of granzyme B during B virus infection but similar expression to mock during HSV-1 infection (Figure 17B). A significant up-regulation is observed in perforin release in HSV-1 infection as compared to mock and B virus infection (Figure 17A). From these results we can conclude that IFN-α and IP-10 might play a role in inducing the release of perforin by NK cells in HSV-1 infections, however, these cytokines might not be important for TNF-α up-regulation. Thus, the absence of IFN-α, among other things, during B virus infection could contribute to lack of NK cell activation.
Figure 17A: Release of cytotoxic granule, perforin by PBMCs during mock, HSV-1, or B virus infection. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.0006. Multiple comparisons between the samples using SNK test shows a significant difference between PBMCs exposed to HSV-1 and PBMCs exposed to mock or B virus treated cells. The error bars represent standard errors.
Figure 17B: Release of cytotoxic granule, granzyme B by PBMCs during mock, HSV-1, or B virus infection. One-way ANOVA of mock, HSV-1, and B virus infected samples yields a p-value of 0.011. Multiple comparisons between the samples using SNK test shows a significant difference between PBMCs exposed to B virus as compared to PBMCs exposed to mock or HSV-1 treated cells. The error bars represent standard errors.
**ITAM receptors are not activated in NK cells or PBMCs during B virus infection.** NK cell activation receptors are divided into three different family members: ITAM, Non-ITAM, and integrins. T-cells, B-cells and NK cells express ITAM activation receptors. In this experiment we wanted to investigate if ITAM activation receptors are induced in NK cells and PBMCs during B virus infection. When an ITAM receptor is engaged by the respective ligand, Syk protein (spleen protein tyrosine kinase) is phosphorylated. ITAM receptors induced activation signal transduction is Syk-dependent. Therefore, in this experiment we measured Syk phosphorylation in NK cells and PBMCs that are exposed to mock infected, B virus, or HSV-1 infected cells. Our western blot results show that Syk is not phosphorylated in PBMCs or NK cells with or without IL-2 treatment (Figure 18). From these results we conclude that if there is any activation observed in NK cells or PBMCs upon infection it is not due to the engagement of ITAM receptors or Syk-dependent activation. We did observe TNF-α production during NK cell exposure to B virus, and this production could be due to Syk-independent activation of NK cells. Also, the expression of cytotoxic granules, perforin, by PBMC upon exposure to HSV-1 infected cells could be the result of Syk-independent activation.
Figure 18: western blot analysis of syk protein phosphorylation in NK cells, NK cells + IL-2, and PBMCs. Syk is not phosphorylated in NK cells with or without IL-2 and PBMCs, thus indicating that syk-dependent activation is inhibited during B virus or HSV-1 infection.
4 DISCUSSION

The close relationship between B virus and human simplex viruses of the same family results in nearly conserved phenotypes and related function. In spite of this, earlier work in our laboratory, coupled with the work performed during this dissertation research showed that B virus lacks a functional domain of a major virus protein, ICP47. The missing domain lead to the speculation that in B virus infected cells virus peptides would be presented via MHC class I molecules on the surface of the infected cells. This speculation, if confirmed would essentially change in a major way a mechanism by which infected cells could be cleared or destroyed by the host immune system when compared to what is known about other simplex viruses. Our hypothesis was that B virus regulated MHC class I antigen presentation differently in human derived cell lines when compared to HSV-1. Thus, at the onset of this project, we sought to verify whether B virus infected cell peptides were presented on the cell surface via MHC class I molecules. In fact, the results of these initial analyses showed that, MHC class I bound viral peptides were presented, albeit in somewhat reduced amounts though not significantly different levels when compared to the levels of MHC class I peptide presentation in cells treated with uninfected cell lysates. The significance of this reduction may be related to several observations that will be subsequently discussed.

During virus infection, excess viral and infected cell proteins are degraded to 8-10 amino acid long peptides by the proteosome. The resultant small peptides bind to the TAP (TAP1 and TAP2 heterodimers) and are subsequently transported to the MHC class I molecule that then
associates with loaded TAP via tapasin. The peptide loaded MHC class I molecule in the endoplasmic reticulum is transported to the cell surface following modification in the Golgi complex. Virus-specific CD8+ T-cells then recognize the peptide-bound MHC class I on the cell surface of the infected cell, bind to the peptide through TCR, and subsequently induce cell lysis. The response of the CD8+ T cells, however, is delayed until these cells arrive at the site of infection.

During alphaherpesvirus infections in humans, the virus down-regulates surface expression of antigen presenting MHC class I molecules, which results in evasion of CD8+ T-cell recognition, but susceptibility to NK cell killing. In the case of HSV infection, this mechanism of down-regulation of surface MHC class I antigen presentation is attributed to ICP47 protein (57, 68, 184). The N-terminal region of HSV-1 ICP47 consists of a TAP binding domain that is also present in HSV-2 ICP47. This TAP binding domain binds to the host TAP and prevents peptide transport resulting in sequestering of unloaded MHC class I in the cytoplasm (68).

In our investigation, we report for the first time that B virus does not down-regulate HLA-A, -B, -C cell surface expression as efficiently as HSV-1. Data analysis suggested there is no significant difference between uninfected and B virus infected macaque-derived cells, MK2 cells, with respect to MHC class I presentation. There is approximately 30% down-regulation of surface MHC class I antigen presentation in B virus infected human-derived cells, HFF cells, however, significantly less than that observed in HSV-1 infected HFF cells (64%). Together, these data suggest that B virus does not down-regulate HLA-A, -B, -C cell surface expression regardless of the host cell line used. In HSV-1 and HSV-2 ICP47 amino acids 3 to 32 make up for the functional TAP binding domain (58), effectively preventing peptides from being loaded onto TAP. B virus ICP47 lacks the conserved TAP binding domain found in both HSV-1 and HSV-2
ICP47 (Figure 19). This domain is not found in any other B virus encoded proteins. Although, B virus ICP47 is a relatively small protein similar to HSV ICP47 and is oriented in a similar manner to HSV ICP47 (126, 137), the lack of TAP binding domain can be predicted to allow for peptide loading onto the cell’s TAP molecule for transport to the MHC class I molecule location. Comparison of ICP47 homolog sequences between human simplex viruses (HSV-1, HSV-2) and non-human primate simplex viruses (B virus, SA8, HVP 2) shows that the latter herpes simplex virus ICP47 diverged from human herpes simplex viruses in that they lack TAP binding domain (Figure 19) (19). Each virus ICP47 homolog’s amino acid sequence length, i.e., HSV-1, HSV-2, BV, HVP-2, and SA8, are 88, 86, 81, 78, and 78, respectively. Perhaps HSV diverged when it added the ICP47 TAP binding domain. The region that binds to host TAP in ICP47 of HSV-1 and 2 is present in the N-terminal region. When ICP47 sequence is compared between all 5 viruses (mentioned above), greater sequence identity is observed in region down-stream to TAP binding domain of ICP47. This observation indicates a function other than MHC class I down-regulation, may be associated with the region that is highly homologous in all the above viruses.

Although down-regulation of MHC-I is characteristic of most human herpesviruses, B virus fails to do so efficiently in cells derived from human or macaques. In contrast, certain human herpesviruses, such as, CMV even encode for more than one protein to down-regulate MHC class I (18), suggesting that down-regulation of MHC class I is an important aspect in evading host adaptive immune responses elicited by CD8+ T-cells as discussed by Goldsmith et al (61). By down-regulating MHC class I during viral infection, viruses prevents CD8+ T-cells from recognizing the infected cells, thus allowing the virus to replicate and spread without interfer-
ence from activated CD8+ T-cells. However, the down-regulation is recognized by NK cells surveying for the presence of damaged cells lacking surface MHC class I antigen presentation.

Figure 19: ICP47 sequence comparison between human herpes viruses (HSV-1 and HSV-2) and simian herpes viruses (B virus, HVP 2, and SA8). Figure A shows the sequence homology between HSV-1, HSV-2, B virus, and HVP-2 ICP47 (1-60 amino acids). The N-terminal region of HSV ICP47 is the TAP binding domain, which is not conserved in B virus ICP47 or in HVP-2 ICP47. The C-terminal region of ICP47, however, is the most homologous region in all the 4 viruses. Figure B shows the phylogenetic tree indicating the ICP47 divergence between human and non-human primate herpes simplex virus. This phylogenetic tree was generated using ClustalW.
The next goal of the research was to determine whether the presentation of cell surface MHC class I molecules loaded with B virus infected cell polypeptides abrogated a major defense observed to exist against human simplex viruses, i.e., destruction of infected cells by natural killer cells. The important role(s) of NK cells in host defenses to reduce HSV-1 induced morbidities coupled with the failure of B virus infected cells to significantly down-regulate MHC class I led us to explore whether the escape of infected cells from primary innate defense strategies could occur.

The down side of down-regulation of MHC class I molecules is that human herpesvirus infected cells are prone to NK cell cytotoxicity, since when present, surface MHC class I molecules act as inhibitory ligands to NK cells. NK cells recognize the MHC class I molecules presented on normal cells and distinguish between “self” and “non-self” or infected and uninfected cells (105, 106, 175). Absence of MHC class I on the cell surface triggers NK cells to kill target cells, and thus, HSV-1 infected cells are sensitive to NK cell lysis (119). At the same time, NK cells require activation ligands on target cells to bind to the receptors necessary for activation. A balance generally exists between activation and inhibitory ligands in order to modulate NK cell activity. Because B virus does not block MHC class I expression on infected cell surfaces, we hypothesized that B virus modulates the infected cell in a manner, which results in protection against NK cell lysis, resulting in critical time for greater local virus replication.

To better understand how B virus may confer protection against NK lysis of the infected cells, it is instructive to evaluate HLA-E and HLA-G, the two isoforms comprising MHC-Ib molecules. MHC-Ib proteins are less polymorphic than MHC-Ia proteins. HLA-E on the cell surface acts as a ligand to CD94/NKG2A, an inhibitory receptor of NK cells. HLA-E molecules are ex-
pressed in almost all nucleated cells and present the leader peptide of classical MHC-Ia molecules (HLA-A, -B, -C). Reduction of HLA-A, -B, -C cell surface expression also reduces surface HLA-E expression. Our results confirmed the direct correlation between HLA-A, -B, -C and HLA-E expression in B virus infected HFF cells, suggesting for the first time that B virus has a mechanism for suppression of NK cell killing, preserving the infected cell to fuse with neighboring cells to facilitate increased virus replication. Since MHC-Ia molecules and HLA-E are expressed on B virus infected cells, infected cell surfaces display, inhibitory ligands to NK cell inhibitory receptors. Is the presence of this ligands sufficient to inhibit NK cell killing of infected target cells?

HLA-G is also an MHC-class Ib molecule, which binds to KIR inhibitory receptor of NK cells. These molecules are mostly expressed on the placental cells (specifically cytotrophoblast cells) to protect the fetus from maternal NK cell induced cytolysis. HLA-G molecules are not expressed on all cells, e.g., fibroblast cells do not express HLA-G under normal conditions. During virus infection, however, HLA-G expression can be induced and expressed on fibroblasts. HLA-G is expressed on the placenta to protect the fetus from maternal NK cell. HLA-G is the least polymorphic of the MHC class I molecules, consisting of 7 isoforms resulting from alternative splicing (116). The membrane bound isoforms, HLA-G1, HLA-G2, HLA-G3, and HLA-G4 are also involved in inhibiting CTL lysis, which is one possible reason for the expression of HLA-G in HSV-1 infected HFF cells. Previous research by Lafon et al., has shown that HSV-1 induces HLA-G expression in neuronal cells, however, their research showed that this HSV induced HLA-G was not expressed on the neuronal cell surface (94). In our experiments, we observed only low-level expression of HLA-G in B virus infected fibroblasts. Nonetheless, with our findings, we predict
that the presence of MHC class I molecules on the surface on B virus infected HFF cells are sufficient to successfully inhibit some, if not all, NK cell activities.

Huard et al. first suggested that lack of MHC class I protein on the cell surface leads to NK cell lysis of HSV-1 infected cells (74). Thus, we suggest here that although NK inhibitory HLA-G molecules were expressed on the cell surface of HSV-1 infected HFF cells, it apparently insufficient to protect the HSV-1 infected HFF cells from NK cell lysis, although this remains to be tested. This study has revealed that B virus, unlike human herpes viruses, does not down-regulate MHC class I molecules on the cell surface efficiently if at all. This also appears to be the case for HVP-2, another closely-related nonhuman primate simplex virus, leading us to believe that B virus, HVP-2, and SA8, and perhaps all non-human primate simplex viruses use a different mechanism to evade infected cell lysis by host immune responses. None of these viruses have TAP binding domains present in the ICP47.5 protein.

Another function of MHC class I molecules is associated with regulation of NK cell activity by binding to the inhibitory receptors (ITIMs), which subsequently result in the phosphorylation of these ITIMs, which then recruit SHP-1 or SHP-2 tyrosine phosphatase, which in turn inactivates the signaling pathway for NK cell cytotoxicity and/or cytokine production (95, 98, 175). Many viruses are known to down-regulate NK cell activity by employing different mechanisms. As discussed previously, CMV can down-regulate expression of ULBP activation ligand by binding and preventing cell surface expression. The MCMV glycoprotein m157 binds to Ly49 receptor of NK cells and inhibits NK cell activity through recruitment of SHP-1 or SHP-2 to phosphorylated ITIM cytoplasmic domains of LY49 (105). Another protein found in both MCMV and HCMV, mimics MHC class I molecules by acting as a decoy that binds to the inhibitory receptors
of NK cells. Specific peptides from UL40 glycoprotein of HCMV bind to HLA-E, which in turn inhibits NK cell activity. HIV nef protein down-regulates HLA-A and –B, but not HLA-C and HLA-E, leading to down-regulation of MHC class I on the cell surface, however, the presence of HLA-E and HLA-C confer resistance to NK cell cytotoxicity (105). KSHV and HCV (glossary) viruses are also known to down-regulate NK cell activity (142). While some virus infected cells are resistant to NK cell lysis, other virus infected cells are sensitive to NK cell cytolysis, which prevents sustained replication of the virus. HSV-1 infected cells are sensitive to NK cell cytolysis due to down-regulation of cell surface MHC class I molecules (74). Vaccinia virus infected cells were also found to be sensitive to NK cell cytolysis, because of the up regulation of cell surface ligands to NK receptors such as, NKp30, NKp44, Nkp46, and NCR (33), each serving as an activating ligand.

Taking into consideration the differences in the modulation of cell surface MHC class I molecules in HSV-1 and B virus infected cells, we hypothesized that B virus does not activate NK cells during infection, escaping NK cell cytolysis and cytokine production, which ultimately leads to increased virus replication at the site of the primary site of infection. In order to test the hypothesis that B virus down modulates NK cell activities, we considered all aspects that determine NK cell activity. To this end, we designed an experimental plan that determines an external effect exerted on the infected cell by NK cells as regulated by infected cells directly or by circulating effector cells (PBMCs), indirectly.

We first quantified MICA and MICB expression in human derived HFF cells infected B virus or HSV-1. The stress proteins MICA and MICB bind to NKG2D activation receptors on the surface of NK cells, as well as other NKG2D bearing cells, and this ligation plays a very important
role especially in overcoming the inhibitory effect of MHC class I cell surface molecules on NK cells. In humans, NKG2D molecules are also present on all CD8+ and γδ TCR+ cells. B virus efficiently blocks cell surface expression of MHC class I molecules as does HSV-1 as shown in the experimental data presented. Additionally, these experiments on MICA and MICB expression demonstrated that surface MICB expression was up regulated at 12 and 24 hpi. When we used a combination antibody anti-MICA/MICB, which recognized both MICA and MICB, to detect cell surface expression, there was increase expression of MICA/MICB on B virus and HSV-1 infected cells. However, when antibodies to only MICA were used in cell sorting experiments, we noted an absence of MICA present on the cell surface of infected cells. From this we concluded that the increase was likely due to the presence of MICB in the absence of availability of anti-MICB specific antibodies. Additionally, ULBP stress proteins are not expressed in uninfected HFF cells, however, they are also not expressed in either B virus or HSV-1 infected cells. Studies on NKG2D ligands on HSV-1 infected HeLa and U373 cells by Schepis et al., previously revealed down-regulation of surface MICA on HSV-1 infected cells, however, MICB was not expressed i(156). There are discrepancies in the results between our study and Schepis et al., study perhaps due to the cell lines used. The cells used in the study presented here are derived from primary fibroblasts, whereas, Schepis group used HeLa cells (transformed cells) and U373 (human glioblastoma-astrocytoma cell line). The fact that different cells express MICA, MICB, ULBP1-4 proteins at different levels (190) has been observed before, therefore there appears to be differences in regulation.
Our data suggests regulation of MICA/ MICB cell surface expression on B virus or HSV-1 infected cells, thus, we next sought to quantify NK cell activity upon their exposure to the MICA/ MICB expressing infected cells. For these experiments we chose naïve NK cells from B virus/HSV-1/HSV-2 antibody-negative individuals, because of the lack of ready availability of fresh blood for same day use from B virus positive donors. Further, we used freshly isolated PBMCs to quantify the specific factors that have the potential to influence NK cell activity. For NK cells to be activated either via cytokines and /or by binding to respective ligands, infected cells should release specific cytokines or chemokines that are requisite for activation and migration of NK cells. NK cell activator, IFN-α, is produced by leukocytes and it helps in activation of NK cells. NK cells are recruited to the site of infection as a result of infected cell release of chemokines, e.g., IP-10, MIP-1a, MIP-1b, MCP-1, and IL-8 (21, 162). Studies on the influence of IFN-α on the NK cell activity during HSV-1 infection have shown that increased IFN-α expression correlated with increased NK cell induced cytotoxicity (119). Experiments on VZV and NK cell activity also showed that NK cell activity is enhanced by IFN-α treatment (114). In our experiments we did not see any expression of migration or activation cytokines (mentioned above) produced by B virus or HSV-1 infected cell at 6 or 24 hpi. Previously, other investigators have shown that during HSV-1 infection there is rapid production of IFN-α by recruited leukocytes and this IFN-α is important for NK cell killing of HSV-1 infected cells (53). In the current studies, treatment of cells with uninfected cell lysates alone induced secretion of specific cytokines, however, B virus effectively blocked the production of these cytokines and chemokines responsible for NK cell activation and recruitment.
To quantify cytokines induced by leukocytes during HSV-1 or B virus infection, we exposed B virus or HSV-1 infected HFF cells to PBMCs to create a cell based in vivo model. Effector: target ratios of 50 : 1 were used in order to ensure the presence of all leukocytes in substantial numbers. There was greater production of IFN-α and IP-10, which are important for NK cell activity in supernatants from cultures containing PBMCs exposed to HSV-1 infected HFF cells, but not from PBMCs exposed to B virus infected cell. Other groups have reported the induction of IFN-α in pDCs during HSV-1 infection (71, 144, 159). Thus, it is clear that PBMCs to produce cytokines necessary for NK cell activity when exposed to HSV-1 infected HFF cells, whereas, B virus appears to have a mechanism to block the production of cytokines even in PBMCs. These results support our hypothesis that B virus down-regulates specific innate immune responses, unlike HSV-1, which has not selected for these blockades. Whether these different pathogenesis patterns account for the fact that B virus causes severe morbidity and often fatal infections in human hosts remains to be studied, however, humans lacking innate immune responses reportedly experience significant morbidities and frequent mortality as a result of HSV-1 infection (118).

To examine to what extent direct activation of NK cells by B virus and HSV-1 infected HFF cells occurred, an effector: target cells ratio of 5:1 was used in similarly designed experiments. For cytotoxicity assays, the effector: target ratio of 5:1 does not yield significant differences in cytotoxicity between mock infected and virus infected cells. We preferred to use this ratio because we did not want to force killing by adding higher numbers of NK cells. Therefore, to quantify NK cell killing of infected cells, we quantified perforin and granzyme B release into the cell supernatants. Because IL-2 plays an important role in inducing NK cell cytotoxicity, we
exposed infected cells to NK cells treated with IL-2 treatment during the course of 18 hrs. We used a final IL-2 concentration of 70 U/ml of culture media during NK cell exposure to infected cells and an initial concentration of 100 U/ml for 1 hr before infection. Prolonged treatment of NK cells with IL-2 drives the NK cells toward synapse formation, subsequent exocytosis of perforin-granzyme lysosome, and target cell cytotoxicity at as low as 10 U/ml (46, 73, 109). We observed no significant release of perforin or granzyme B by NK cells upon exposure to B virus or HSV-1 infected HFF cells, suggesting that neither virus induces direct killing by naïve NK cells even with IL-2 treatment. Virus selection of traits that promote NK cell induced cytolysis of infected cells would not be beneficial for virus survival within a host. On the other hand, selections of traits that reduce virus replication and ensure host survival are beneficial to the virus long-term survival. Unlike B virus, HSV-1 infections in humans result in minimum morbidity and the virus can utilize an individual host for the entire lifetime of that individual.

As a result of the experiments designed for this study, we observed that TNF-α was produced during B virus infection upon exposure of infected cells to NK cells, suggesting the presence of cell surface ligands that bind to NK cell that result in the production of TNF-α. Further, IL-2 treatment of NK cells induces an increase in TNF-α production over and above that resulting from NK cell receptor: ligand binding to infected cells. Another possibility is that the TNF-α produced during B virus infection might be produced by undifferentiated NK cells as the amount of TNF-α produced was below the functional TNF-α concentrations (139).

Because there was no observed activation of NK cell cytotoxicity resulting from direct interaction of infected cells with NK cells, and since we observed the production of IFN-α and IP-10 by PBMCs exposed to infected cells, we next sought to identify PBMC-activated NK cells. To
accomplish this, we exposed infected cells to PBMC at 50:1 effector: target ratio and determined NK cell cytotoxicity by measuring perforin and granzyme B release. Generally, NK cell (during primary infection) and CTL populations (during secondary infection) contained in PBMCs primarily release perforin and granzyme B. Because we used virus antibody negative donors, CTL cell populations would not be activated upon exposure to B virus or HSV-1 infected cells, and thus perforin and granzyme B released can be attributed to NK cells only. In the experiments presented here, we observed increased perforin in supernatants collected from cultures containing PBMCs exposed to HSV-1 infected HFF cells compared to mock infected cells, however, there was no observed increase in perforin and granzyme B in the supernatants obtained from B virus infected HFF cells exposed to PBMCs. These results suggest that NK cells within the PBMCs were not exposed to IFN-α and IP-10 during B virus infection, while naïve NK cells without previous exposure to infection remained inactive even when stimulated with IL-2. Thus, B virus infected cells do not stimulate NK cell responses probably as a result of the B virus specific blockade of cytokine and chemokine production necessary for NK cell activation as well as the preservation of MHC class I surface antigen presentation. B virus infected cells do, however, have a positive effect on TNF-α production indirectly.

The production of TNF-α by NK cells during B virus was not observed to be regulated by Syk-dependent pathway and therefore a non-ITAM receptor-ligand induced pathway is most probably responsible e.g., DAP10:NKG2D. Similarly in case perforin and granzyme B release by PBMC activated NK cells exposed to HSV-1 infected cells, the NK cell activity is not through ITAM receptor activation.
Figure 20 and 21 illustrate NK cell activity during B virus infection and the parallel effects of HSV-1.

**Figure 20: NK cell activity during HSV-1 infection.** HSV-1 infected HFF cells down regulate or do not induce cytokines and chemokines needed for NK cell activity. PBMCs exposed to HSV-1 infected cells produce IFN-α and IP-10, which in turns help in NK cell activation in the context of PBMCs.
**Figure 21: NK cell activity during B virus infection.** B virus infected HFF cells or these cells exposed to PBMCs down regulate or do not induce cytokines and chemokines needed for NK cell activity. Thus, B virus down regulate NK cell activity to a large extent except for the production of TNF-α.
In ICP47 deletion mutants, HSV is less neurovirulent as a result of infected cell destruction by CTLs. Thus, HSV-specific CD8+ T-cells do play an important role in eliminating HSV infected cells, and thus, reducing neurovirulence, however, they are not a primary defense mechanism early in infection. Virus-specific CTLs also play a similar role during secondary infection (61, 103, 171). Due to stress or other factors resulting in suppression of immune response, HSV specific CD8+ T-cell responses can be impaired and virus can reactivate (11, 14, 55, 120). In the case of B virus, infected cells can activate CTLs, yet neurovirulence is striking in zoonotically infected humans. How then does B virus control CTL destruction at the primary site of infection? Does suppression of NK responses allow sufficient time for virus to replicate, such that the effect of CTLs at a later time point does not significantly reduce virus load.

Natural killer cell lysis of infected cells leads to controlled virus replication thereby preventing the rapid spread of virus. For this reason NK cells were implicated in HSV-1 infection since their absence correlated to increased morbidity e.g., encephalitis. Mouse studies by Alder et al have suggested that NK cells provide protective immunity from mortality due to HSV-1 encephalitis in mice lacking T-cells (5). This is particularly important because in human HSV-1 infection, CD8+ T-cells are rendered inactive due to lack of surface MHC class I antigen presentation.

Activated natural killer cells are also capable of interacting with dendritic cells, ultimately enhancing the efficiency of adaptive immune response. Therefore, the presence of activated NK cells is not only important for controlling virus replication, but also important for eliciting robust adaptive immune responses. Because of the observations that B virus infection often does not result in a strong humoral immune response, especially in individuals who suc-
cumb to infection, we find it interesting to speculate that natural killer cell activity is important for minimizing B virus pathogenesis. Whether natural killer cell populations are compromised in individuals who succumb to zoonotic infection remains to be investigated.

Observations from our studies provide the basis for further examination of NK cell activity using cell cytotoxicity assays that will help elucidate the role of NK cells. Factors that induce cell death by NK cells other than the release of perforin and granzyme B include TNF-α and Fas-FasL induced apoptosis. Future studies will focus on identification of CD8+ T-cell specificity directed toward B virus infected cells and whether CTL activity against B virus infected cells effectively limits virus replication. Future studies will allow us to explore different immune evasive strategies employed by B virus that influence rapid spread of virus to the central nervous system and the effect of extant immunological defenses on the pathogenesis of zoonotic infection.
5 CONCLUSIONS

Previous results from our lab suggested that B virus infected cells do no mount robust innate immune responses (Zao, et al., submitted, 2009). B virus infection in HFF cells does not interfere with host MHC class I antigen loading and cell surface expression, unlike HSV-1 infected HFF cells. Our data suggest that CD8+ T-cell recognition of MHC class I presenting B virus antigen on the infected cell surface has the potential to cytolise infected cells later in infection, since these cells are not present early in infection pathogenesis. This exploration was based on the rationale that B virus lacked the ability to block peptide loading onto MHC class 1 molecules. In contrast, by blocking MHC class 1 peptide loading via ICP47, HSV can escape cytolysis by CD8+ CTLs. In doing so, however, infected cells can be quickly killed by NK cells on patrol for cells not expressing these critical MHC class 1 molecules. These events do occur very early in infection, within days of the onset of infection. MHC class I antigen expression on the B virus infected cell surface indicates clearly that B virus employs a different strategy from HSV-1 in evading host innate and adaptive immune responses. MHC class I antigen expression on the cell surface negatively regulates NK cell activity, therefore, we suggest from our studies that B virus is insensitive to host natural killer cell defenses. Naïve NK cells upon exposure to B virus infected cells did not release any cytotoxic granules (perforin and granzyme B), while these cells did produce TNF-α, they did not produce any other cytokines such as, IFN-γ and GM-CSF. Further, our results suggest that B virus infection does not result in the release of cytokines or chemokines necessary for NK cell activity even with contributions from PBMCs, whereas, HSV-1
infected HFF cells exposed to PBMCs induce IFN-α and IP-10 that can further enhance NK cell activation, already in motion due to the lack of MHC Class 1 antigen expression, which is blocked by ICP47 TAP-binding domain. These results suggest that NK cells during B virus infection are not functional. Thus, B virus down regulates NK cell cytotoxicity and cytokine activity in order for the virus to replicate and spread rapidly to other cells. Non-functional NK cells were found to be one of the causes for HSV encephalitis and also other encephalitis causing viruses. Therefore, from our results we speculate that CNS disease severity in fatal B virus infection could be a result of non-functioning NK cells.
6 REFERENCES


