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B Virus Circumvents Innate Responses in Human Cells

Chih-Ling Zao

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by

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ABSTRACT

B virus (*Cercopithecine herpesvirus 1*) is an alphaherpesvirus indigenous to macaque monkeys and is closely related to herpes simplex virus type 1 (HSV-1). Disease caused by B virus, which is often mild or asymptomatic in its natural host, the macaque monkey, is similar in infected macaques to HSV-1 infection in humans. When B virus zoonotically infects foreign hosts, e.g., humans, high morbidity and mortality are evidenced in > 80% of untreated cases. To explore the underlying reasons for differences in pathogenesis between B virus and HSV-1 infection in humans, human microarrays were used to comparatively examine global cellular gene expression patterns engaged as a result of infection of human foreskin fibroblasts (HFFs). Our results demonstrate that these closely related simplexvirus family members have divergent strategies to thwart host cell pathways related to innate defenses. In these studies, B virus did not induce detectable interferon, cytokine or chemokine genes, in sharp contrast to HSV-1, which induced innate immune responsive genes in infected cells. Although no innate immune response genes were found to be up-regulated by B virus infection, B virus induced IκBζ, which was the only gene found to be involved in the NF-κB signaling pathway

within the innate immunity biological network. Quantification of NF-κB p50 DNA binding activity in virus-infected nuclear extracts demonstrated that NF-κB p50 DNA binding activity was lower in B virus-infected cells. Suppression of IκBζ in B virus infected cells by siRNA restored NF-κB-induced cytokine and chemokine expressions. Data presented here support the model that IκBζ inhibits NF-κB regulated immune responsive genes in B virus-infected HFF cells, and this response differs from that observed in HFF cells infected with HSV-1. The result is that B virus alters the NF-κB regulated expression of cytokine and chemokine genes of HFF cells differently from HSV-1 early after infection. These differences in cytokine and chemokine expression may be associated with the delayed or reduced host responses observed in B virus infected humans and underlie the failure of adaptive responses in zoonotically infected humans.

INDEX WORDS: B virus, innate immunity, HSV-1, microarray, NF-κB, IκBζ

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DEDICATION

To my family, specially my mother and father,

for their unconditional love and patience.

Also to my heavenly Father,

for His amazing love and blessings.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AP1	activator protein 1
APCs	antigen-presenting cells
BCL3	B-cell CLL/lymphoma 3
BMBL	Biosafety in Microbiological and Biomedical Laboratories
DAG	diacylglycerol
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagles minimum essential medium
FDR	false discovery rate
fMLP	N-formylated peptides
gD	glycoprotein D
HBSS	Hanks balanced salt solution
HFF	human foreskin fibroblast
h	hour
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICP	infected cell protein
IFN	interferon
IκB	inhibitor of kappa light polypeptide gene enhancer in B cells
IKK	IκB kinase
IL	interleukin

IP_3	inositol-1,4.5-trisphosphate
JAK	Janus kinase
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
LTB4	leukotriene B4
MAPK	mitogen-activated protein kinase
MEKK	mitogen-activated protein kinase
MHC	major histocompatibility complex
m.o.i.	multiplicity of infection
MPO	myeloperoxidase
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B cells
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta
NIK	NF-κB inducing kinase
NK cells	natural killer cells
PBS	phosphate buffer saline
$PI(4,5)P_2$	phosphatidylinositol-4,5-biphosphate
PKB or Akt	protein kinase B
PKC	protein kinase C
PMNs	polymorphonuclear granulocytes
p.i.	post infection
PTKs	protein tyrosine kinases
RMA	Robust Multichip Analysis

RHD	Rel Homology Domain
SD	standard deviation
SOCS3	suppressor of cytokine signaling-3
STATs	signal transducers and activators of transcription
Sup.	Supernatant
TGF-β	transforming growth factor
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor-α
VP	virion polypeptide

SECTION I: INTRODUCTION

Part 1. Literature Review

An overview of B virus.

B virus is named after the first B virus-infected patient, W.B., who was bitten by a monkey in 1932 and died from progressive encephalomyelitis 18 days later (139). Various names have been ascribed for this virus in the literature, including *Cercopithecine herpesvirus 1, Herpesvirus simiae,* monkey B virus, herpesvirus B and herpes B. It was the first nonhuman primate herpesvirus identified. The virion consists of a core containing a linear double-stranded DNA of about 157 kb (123), an icosadeltahedral capsid about 160-180 nm in diameter, an amorphous tegument surrounding the capsid, and an envelope exhibiting viral glycoprotein spikes on its surface (133) (92). It is endemic in Old World monkeys and usually causes asymptomatic or mild infection in these natural host animals. Only about 50 cases of B virus infection in humans have been identified so far. However, the death rate for B virus-infected humans was >80% before the advent of antiviral therapy (166) (30) .

Classification and characterization of B virus

Classification and nomenclature. B virus belongs to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, and *Simplexvirus* genus. *Herpesviridae* has three subfamilies: a*lphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*. The members of *alphaherpesvirinae* have variable host ranges, relatively short reproductive cycles, efficient destruction of infected cells and the capacity to establish latency primarily in sensory ganglia. The *betaherpesvirinae* subfamily has a restricted host range, long reproductive cycle, and causes latent infection in secretory glands, lymphoreticular cells, kidneys, and other tissues. As for the *gammaherpesvirinae*, its host range is limited to the natural host. *In vitro*, all members of this subfamily replicate in lymphoblastoid cells. Because the biological, molecular and serological properties of B virus are similar to other members of alphaherpesviruses, herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) of humans, the International Committee on the Taxonomy of Viruses designated B virus as *Cercopithecine herpesvirus 1* (131).

Biological properties. The growth properties of B virus in natural hosts resemble HSV-1 and HSV-2 *in vivo* (135) (137) (136) (23). In cell culture, B virus also has similar replication kinetics as HSV-1 and HSV-2 (166), although B virus replicates slightly more rapidly. Virus adsorption to susceptible cell surfaces occurs within 30 to 60 minutes, and an eclipse period of cell and viral activities is followed within 2 to 3 hours. The non-enveloped capsid is transported into the nucleus where viral DNA genome is released.

Viral DNA replication takes place in the nucleus at approximately 4 hours (h) post infection (p.i.). Cellular proteins as well as virion components are required for viral DNA replication in permissive cells. Like other herpesviruses, the expression of five viral gene groups, α, β1, β2, γ1,and γ2 are tightly regulated. Progeny B virus can be detected as early as 6 hr p.i. (67).

The double-stranded genome of B virus strain E2490 is composed of 156,789 bp, slightly larger than HSV-1 (152kb) and HSV-2 (155kb) (123). The guanosine and cytosine (G+C) content is 74.5%, which is as high as HSV-1 and HSV-2. The genome structure contains unique long (UL) and unique short (US) regions flanked by a pair of inverted repeats, inverted long repeats (RL) and inverted short (RS) repeats, separately. The repeats allow rearrangements of the unique regions, and thus, the virus genome can exist as a mixture of four isomers. There are 74 genes identified from the complete B virus genome with the amino acid identity between B virus and HSV proteins ranging from 26.6 to 87.7%. Because many of these viral polypeptides share common antigenic determinants, antibodies induced by B virus or HSV-1 are highly cross-reactive (66) (43). This makes serological diagnosis of B virus infection in human cases complicated when the patients are also HSV-1 and HSV-2 seropositive.

Host range and pathogenic properties

Initial isolation of B virus. B virus was first isolated from a 29-year-old physician (W.B.) who died from the bites of a healthy-appearing *Macacus mulatta* monkey (rhesus) in 1932 (55) (139). Three days after the bites, the wounds on the patient's left little and ring fingers became inflamed, and subsequently regional lymphangitis and lymphadenitis developed. The patient was admitted to the hospital 6 days later with a fever of 101.4°F. Several small cloudy fluid-filled vesicles formed near the wounds over the next few days. Generalized abdominal cramping developed 10 days after infection and followed by marked hyperesthesia of the lower extremities associated with urinary retention 13 days after monkey bite. Analysis of the CSF indicated mononuclear pleocytosis along with an increased level of albumin and globulins. The patient's condition deteriorated over the following days, as indicated by hyperesthesia of the upper extremities and respiratory paralysis. Death occurred 5 hours after the patient lapsed into a coma 18 days after infection.

The histological diagnosis was acute transverse myelitis with areas of focal necrosis and hemorrhage in the regional lymph nodes, spleen, and adrenal glands. Inflammatory exudates, composed of mononuclear cells, were noted in the gray and white matter of the spinal cord. Additional lesions in the medulla, pons, basal ganglia, internal capsule, uncinate gyrus, and frontal lobe were composed of perivascular mononuclear cell infiltrates. The cause of death was listed as encephalomyelitis. Two

research groups, led by Gay and Sabin (55) (139), obtained samples from patient W.B. and both reported the existence of an ultrafilterable agent recovered from neurological tissues that was lethal to rabbits but not rhesus macaques. The pathologic manifestations of the agent in diseased rabbits demonstrated many features in common with those in the human disease. Gay and Holden reported the agent to be antigenically related to HSV and designated it "W virus" after the first initial of the patient. Sabin and Wright designated the isolated "B virus" after the second initial of the patient. The name B virus was widely adopted ever since.

Human B virus infection. To date, fewer than 50 human cases of B virus infection have been documented. Two thirds have occurred in the United States and the others have been reported in Canada and Great Britain. The actual number of human cases may be underestimated, particularly if hypothesized asymptomatic cases are considered (91) (11) (70) (51), and in addition, the lack of confirmed human cases in Asian countries where humans and monkeys have a high contact frequency. The well documented routes of B virus infection in human cases include monkey bites (37) (139) (18) (73) (20) (119) (70) (36), monkey scratches (37) (91), exposure to tissue culture-bottle cuts (73), exposure to the tissues during autopsies of monkeys (37), wound contamination with monkey saliva (138), cage scratch (70) (149), needlestick injuries (37) (5), mucosal splash (127), human-to-human contact (70), possible aerosol (113) (72), and possible reactivation of B virus (47).

Human B virus associated disease generally occurs within 1 month after exposure with an incubation period of a few days to weeks (69), but this can occur after even years have passed (47). The development of disease is thought to depend on the site of exposure and the amount of virus inoculated. Vesicular lesions have not been consistently found at the site of exposure. Patients often start with general flu-like symptoms of fever, muscle aches, fatigue and headache. Other variable symptoms include regional lymphadenitis, lymphangitis, nausea, vomiting, abdominal pain and hiccups. Neurological signs develop when the virus spreads along the nerves of the peripheral nervous system (PNS) to the central nervous system (CNS). Once the spinal cord or brain is infected by the virus, most patients die even with antiviral therapy intervention. Deaths are often attributed to respiratory failure associated with ascending paralysis (119) (70) (161). The possibility of asymptomatic or mild B virus infection in humans has been suggested, but it is considered rare if it occurs at all (51). Nonetheless, one case in which the disease occurred years after the initial exposure suggests that B virus is capable of establishing latent infection in humans (47).

B virus infection in natural hosts, macaques. Besides humans, B virus infects a broad range of mammalian species, including Old World monkeys and New World monkeys. Old World monkeys of the genus *Macaca* are natural hosts of B virus, and almost all of this genus naturally exist in Asia. The most commonly reported B virus infections are found in the rhesus and cynomolgus macaque (*M. Facicularis*), two species used extensively in biomedical research. Other macaque monkeys including stumptail (*M.*

artoides), pig-tailed (*M. nemestrina*), Japanese (*M. fuscata*), bonnet (*M. radiata*), and Taiwan (*M. cyclopis*) have also been reported to naturally carry B virus (161). The prevalence of serum-detected B virus-induced antibodies in both wild and captive adult macaques can reach up to 70-100% (145) (117) (119) (162) (69). The routes of transmission are primarily through sexual activity and bites.

Like HSV infection in humans, B virus infection in monkeys is life-long and can remain latent in the sensory ganglia. Intermittent reactivation and shedding of viruses in saliva or genital secretions may occur, particularly during periods of stress or immunosuppression (161). Most infected macaques are asymptomatic. Oral herpetic lesions similar to HSV infection in humans, such as gingivostomatitis, oral and lingual ulcers, and conjunctivitis have been described, which usually heal spontaneously in 7 to 14 days (161). In rare instances, systemic illness has even been reported, including the occurrence of ulcerative lesions in the mouth, esophagus, and stomach; diffused necrosis of the liver, spleen, and adrenal glands; cerebral infarction; interstitial hemorrhagic pneumonia and focal hepatitis, but most of these cases appear to be associated with immunosuppression (96) (34) (45).

Natural host immune responses

Host immunity in B virus infection. The host immune responses induced by B virus infection are far from being fully understood, but there is significantly more knowledge

of humoral antibody responses than of innate or cellular immune responses. Localized infection in skin and muscles usually results in vesicular rash in both the natural and foreign hosts. Virus infection in natural hosts usually heals spontaneously in 7 to 14 days, and virus spread is facilitated via travel through the PNS to enter the sensory ganglia for life-long latency. B virus infection in foreign hosts, however, can cause focal necrosis and hemorrhage in regional lymph nodes after virus exposure in the primary infected sites. Virus travels to the CNS by way of the PNS, centralizing in a small region of the cervical spinal cord causing focal lesions. In some human cases, dissolution of the cord in the region where the virus resides has been observed (139) (119) (161) (166). Whether the destruction is due to an immune response, lytic replication, or a combination of both remains unclear. Although little is known about cell-mediated immune responses induced by B virus infection in both natural and foreign hosts, a humoral immune response, initially IgM production, can be detected within 7 to 10 days p.i. with IgG subsequently appearing on day 14 p.i. in macaques (85) (166), but in zoonotically infected humans these responses appear to be delayed and at lower levels. Neutralizing antibodies appear both in the natural and foreign hosts, but at significantly lower titers in foreign hosts. The cause of this disparity in antibody responses is not known presently.

Host innate immunity in HSV-1 infection. Immunological knowledge gathered from HSV-1 studies provides a paradigm to understand B virus pathogenesis, because these two viruses are evolutionarily closely related. In the case of HSV-1 infection, responding host defense includes both innate and adaptive immunity. Immune effector cells

including tissue macrophages, natural killer (NK) cells, circulating monocytes, polymorphonuclear granulocytes (PMNs) together with cytokines, provide nonspecific innate immune responses as a result of HSV-1 infection (168). Interestingly, hosts that lack the ability to mount an innate response suffer a high morbidity when infected with HSV.

Resident macrophages are initially activated when HSV-1 first penetrates into the epithelium. Cytokines and chemokines secreted by the activated macrophages, especially cytokine tumor necrosis factor-α (TNF-α), induce numerous changes in endothelial cells of nearby blood capillaries. Cytokines induce the cell adhesion molecules, P-selectin and E-selectin, to appear on the endothelial cell surface, causing PMNs (mainly neutrophils) and monocytes to adhere to and roll on the endothelial surface in large numbers. Cytokines also induce the production of the intracellular adhesion molecules (ICAMs and VCAM-1) on endothelial cells, which bind to adhesion molecules on leukocytes and strengthen interactions between leukocytes and endothelial cells, as well as help neutrophils and monocytes to enter infected tissue in large numbers to form inflammatory foci. As monocytes mature into tissue macrophages and become activated, more and more inflammatory cells are attracted into the infected tissue and the inflammatory response is maintained and reinforced (76).

NK cells form a lymphocytic subset that express cell surface antigen CD56+. They are large granular lymphocytes that develop from bone marrow and circulate in

blood before localizing in specific tissues. The cell killing function of NK cells is mediated by the release of cytotoxic granules to the surface of bound target cells leading to the induction of programmed cell death. NK cells have two types of surface receptors to control their cytotoxic activity besides Fas-Fas Ligand mediated cytotoxicity (4). One type is an activating receptor (or killer receptor), which triggers killing by NK cells as a result of carbohydrate recognition on many cells. In HSV-infected cells, target structures for NK cell recognition and induced lysis of infected cells are structural viral glycoproteins, nonstructural viral proteins or virally induced cellular gene products during the early course of an infection (13) (48). The other type of receptors on NK cells are the killer inhibitory receptors, which can inhibit signals induced from activated receptors, and thus prevent activation of NK cells. Killer inhibitory receptors are specific for MHC (major histocompatibility complex) class I alleles, which help to explain why NK cells selectively kill target cells bearing low levels of MHC class I molecules. Because altered or absent MHC class I molecules cannot stimulate negative signals, NK cells can be activated by activating signals. NK cells are also activated in response to many cytokines, such as IFN-α/β produced by infected cells, dendritic cells or macrophage-derived cytokines, e.g., interleukin (IL)-12 and TNF-α. The activated NK cells secrete interferon (IFN)-γ, which is crucial in controlling infections before T-cells have been activated to produce IFN-γ. Cytokines with the potential to function in the negative regulation of NK cells are IL-10 and transforming growth factor (TGF)-β. IL-10 produced by Th2 cells can inhibit induction of IL-12 from dendritic cells and macrophages, whereas TGF-β from Th2 cells production can block NK cell proliferation

and cytotoxicity as well as inhibit induction of IL-12 and NK cell IFN-γ production (12).

Many cytokines produced during innate immune responses, especially the IFN families, may dictate host cell susceptibility to virus infection by limiting virus replication and spread. There are three categories of interferons: IFN-α, IFN-β and IFN-γ (75). The two type I IFNs, IFN-α (leukocyte IFN) and IFN-β (fibroblast IFN), are synthesized by many cell types following infection by viruses and are very different from type 2 IFN-γ which is produced by CD4+ Th1 cells, CD8 T cells, and NK cells (158). All the IFNs share similar signaling pathways that begin with a ligand-receptor interaction, followed by activation of tyrosine kinases of the Janus kinase (JAK) family that phosphorylates substrate proteins called STATs (signal transducers and activators of transcription). These STATs subsequently form homomeric or heteromeric protein complexes that translocate to the nucleus and bind to specific DNA sequences in the promoter regions of stimulated genes (89). Antiviral activities and immunomodulatory actions caused by IFNs provide an early line of defense against specific viral infections. The effect of IFNs is to block HSV-1 replication primarily in the early stages of infection during the onset of immediate early α mRNA transcription (116). IFNs induced directly by virus infection or indirectly via other cytokines, e.g., IL-12, can trigger effective cellular defenses including NK cell activity. In addition, they may influence subsequent adaptive immune response (12).

Other cytokines produced within herpetic lesions in epidermal cells and infiltrating immune cells are also critical for the control of virus infection. In herpetic vesicles, high concentrations of β-chemokines, IL-12, and IFN-α, β, γ are detected (103) (154). The local release of β-chemokines at the site of infection recruits monocytes and T-lymphocytes to the infected tissues. IL-12 activates NK cells and induces differentiation of CD4+ T cells into Th1 cells. Macrophages also secret IL-12 and other cytokines, such as interferon, IL-1, IL-6, IL-8 and TNF-α within the lesions. CD4+ T lymphocytes may secrete a Th1 pattern (IFN-γ and TNF-α) or a Th2 pattern (IL-10, TGF-β) of cytokines (103). Th1 patterns lead to activation of cytotoxic T cells while Th2 patterns lead to production of neutralizing antibodies (33).

Induction of adaptive immune responses, which includes antigen-specific T and B lymphocyte responses, begins with activation of dendritic cells. Dendritic cells are potential professional antigen-presenting cells (APCs) residing in most tissue. Dendritic cells mature into potent APCs when they take up pathogens, migrate from infected tissue in lymph to secondary lymph nodes, from which adaptive immunity is initiated. Activated dendritic cells secrete cytokines, such as IFN-α, IL-1α, IL-1β, IL-6, IL-10, IL-12, TNF-α, and GM-CSF (56), that influence both innate and adaptive immune responses. Adaptive immune responses are initiated when naïve T cells in lymph nodes recognize antigens on surfaces of dendritic cells and become activated. Naïve T cells then mature into effector CD4+ Th1 cells or CD8+ T cells, re-enter circulation and migrate to local sites of infection.

Inflammatory and neutrophil chemotaxis

Inflammatory. The inflammatory response is part of innate immunity. It occurs when tissues are injured by trauma or infection. Chemicals including histamine, bradykinin and serotonin are released by damaged tissue. These chemicals cause dilation and increased permeability of blood vessels during inflammation, which lead to increase in local blood flow and the leakage of fluid that account for the heat, redness, and swelling. TNF-α, IL-1, and IL-6 are the classical pro-inflammatory cytokines secreted from phagocytic cells. Locally, they act together with chemokines to attract inflammatory cells to migrate from circulation to the sites of infection and account for the pain. The main inflammatory cell types seen in initial phases are neutrophils, which engulf and destroy invading pathogens. The influx of neutrophils is followed by monocytes that rapidly differentiate into macrophages. Inflammatory responses later in an infection also involve lymphocytes, which have meanwhile been activated by antigens that have drained from the site of infection via the afferent lymphatics. When the causes of the inflammatory reaction are of a high intensity, the production of cytokines is increased, and they are released in the circulation provoking the "acute phase response". On the other hand, "inhibitory" cytokines, such as IL-10, dampen the activation of some effector functions of T lymphocytes and mononuclear phagocytes, by inhibiting the release of pro-inflammatory cytokines, thereby turning off the inflammatory processes (27).

Neutrophil. Neutrophils are the most abundant white cells in the blood. They were historically called microphages due to their smaller sizes compared with macrophages. They are also a type of granulocytes, and, together with eosinophil and basophils, are called polymorphonuclear leukocytes (PMN) because of their irregularly shaped nuclei. Neutrophils are not resident in healthy tissues, but rapidly migrate from blood to the sites of tissue damage or infection. The arrival of neutrophils is the first series of inflammatory response, by which cells and molecules of innate immunity are recruited into sites of infection. Neutrophils themselves release toxic proteases, phagocytize pathogens, and generate reactive oxygen intermediates and hydrogen peroxide, promoting the destruction of invading pathogens (86). Therefore, they are at the front line of innate immune defense to engulf and kill microorganisms.

Neutrophil chemotaxis. Chemotaxis is the process by which cells can detect and migrate up a chemoattractant gradient (173). In the initial inflammatory stage, circulating blood neutrophils adhere to endothelium in response to proinflammatory factors (e.g. cytokines, chemokines, TNF-α, IL-1 and Lipopolysaccharide {LPS}) at the endothelial interface and then transmigrate through the vessel wall into tissues by sensing chemoattractant gradients secreted from nearby macrophages, mast cells and infected cells. Potent neutrophil chemoattractants include bacterial products, such as N-formylated peptides (fMLP), host-derived products, such as IL-8, growth-related peptides α (GROα, GRO-1, MGSA, NAP-3, CXCL1), growth-related peptides β (GROβ, GRO-2, MGSA, MIP-2α, CXCL2) and growth-related peptides γ (GROγ, GRO-3, MGSA, MIP-2β, CXCL3),

epithelial cell-derived neutrophil-activating factor 78 (ENA-78), granulocyte chemotactic peptide 2 (GCP2, CKA-3, CXCL6), neutrophil attractant protein 2 (NAP-2, EN-78), leukotriene B4 (LTB4), platelet-activating factor, lipoxins; products of immune activation, such as complement fragments C3 or C5a (40, 173). Chemokines (such as IL-8 and LTB4), which allow neutrophils to move to the surrounding areas of infections or tissue damage were called intermediary chemoattractants (24). Once neutrophils have been attracted to the nearby inflammatory sites, they would then follow a series of end target chemoattractants (such as fMLP or C5a) to the final site of infection (64).

Signaling pathways for neutrophil chemotaxis. Neutrophils respond to chemoattractants by G protein (heterotrimeric GTP-binding regulatory proteins)-coupled receptors on their cell surfaces. Ligation of chemoattractants to these seven-transmembrane helix receptors triggers the exchange of GTP for the GDP on the α subunit of heterotrimeric G proteins and dissociation of α subunit from the β γ subunit pair (167). Free Gα subunit interacts with phosphatidylinositol-specific phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-biphosphate PI(4,5)P_2 to generate inositol-1,4.5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 further mediates the release of intracellular Ca^{+2} from the endoplasmic reticulum and DAG mediates the activation of protein kinase C (PKC) (28). Activated PKC subsequently results in neutrophil transmigration, including cell adhesion, cytoskeletal reorganization, and actin filament polymerization, to push the plasma member forward and induce crawling (90). On the other hand, dissociated $\beta\gamma$ subunit complex of G-protein coupled receptors interact with

PI3-kinase, which is responsible for converting member $PI(4,5)P_2$ into $PI(3,4,5)P_3$ (29). The accumulation of $PI(3,4,5)P_3$ on the cell membrane determines the leading edge of the cell polarity and motility toward chemotactic gradients (159). PI3-kinase also activates protein kinase B (PKB or Akt). Upon PI3-kinase activation, PKB (Akt) is recruited to the plasma membrane, where it undergoes phosphorylation. The activation of PKB (Akt) correlates with the direction of membrane protrusion and the underlying actin polymerization (144). The intermediary chemoattractants (IL8 and LTB4) primarily function via this PI3K/Akt pathway after they interact with G protein coupled receptor on the cell surface. End target chemoattractants (fMLP and C5a), however, function primarily by dissociated $G_{\beta\gamma}$ subunit complex activating PI3Kγ leading to p38 mitogen-activated protein kinase (MAPK) activation (64).

Besides the classical G-protein receptor-coupled signaling pathways described above, neutrophil chemotaxis can also be mediated by protein tyrosine kinases (PTKs). However, the steps that follow the activation of the G protein-coupled receptors and link them to the modulation of the activity of tyrosine kinases remain unclear. Among more than 10 subfamilies of PTKs, several PTKs involved in neutrophil transmigration signaling transduction have been identified, including Fgr, Hck, Lyn, Yes and Syk (163) (128) (107). Other PTK, such as Tec family tyrosine kinases, Btk, Tec and Bmx, are also involved in the regulation of neutrophil transmigration (82).

Innate immunity signaling pathways

It is unclear if B virus induces cellular signaling pathways to regulate these host innate immune responses, but studies using HSV-1-infected cells have demonstrated that several cellular signaling pathways are up-regulated to activate transcription factors that ultimately coordinate immune responsive gene expression during the course of HSV-1 infection (Table 1). Based on experimental evidence, some investigators suggest that HSV-1 utilizes glycoprotein D (gD) binding to the HveA receptor to activate the transcription factors NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B cells) and AP-1 (activator protein 1, c-Jun, p39) (93) (108) (3) (83) (98). The activation of NF-κB by HSV-1 can also depend on virus penetration and/or immediate early gene expression of ICP4 (Infected Cell Protein 4) and ICP27 (122) (2). Other cellular signaling pathways by which HSV-1 can induce transcription factors AP-1 and ATF-2 (activating transcription factor-2) involve ICP0 and/or VP16 (Virion Polypeptide 16) to activate MAPK family, Jun N-terminal kinase (JNK) and p38 pathways (77) (174) (97). Additionally, the activation of MAPK pathway can be potentially dependent on HSV-1 glycoprotein H (gH) binding to induce JNK activity (52).

Table1: HSV induced signal transduction pathway review.

Virus	Cell line	Viral gene	Signaling pathway	Induced cytokine	Reference
HSV-1	RAW264.7 (murine macrophage) NIH3T3 (murine fibroblast)	ICP0	PKR NF-κB IRF3	RANTES/CCL5	(99) (100)
HSV-1	Human PBMC	gD		IFN-α	(3)
HSV-1	Human PBMC	gD		IFN-α	(62)
HSV-1	RAW264.7	gD Early post-entry event dsRNA-PKR		TNF-α (within 2-24 h)	(103)
HSV-2	RAW264.7 C57BL6/ peritoneal cells	gD	NF-κB ATF2/Jun (transcriptional regulation) (1h p.i.) AU-ruch region of 3' untranslated region (translational regulation)	TNF-α	(100, 121)
HSV-2	Murine macrophage			IFN-α/β TNF-α	(26)
HSV-1	Purified DC from human PBMC			IFN-α, IL-1α, IL-1β, IL-6, IL-10, IL-12, GM-CSF, and TNF-α within 4 to 12 h IFN-α and IL-6 within 24 h	(44, 56)

Virus	Cell line	Viral gene	Signaling pathway	Induced cytokine	Reference
HSV-1	C57BL/6Cr Corneas and TG			IL-1α, IL-5, IL-6, IFN-γ (2-7 days p.i.)	(48)
HSV-2	BALB/c			IL13	(101)
	HSE-1 (Herpes simplex encephalitis type I) patient CSF and serum			IL-8, macrophage inflammatory protein 1 (MIP-1), monocyte chemoattractant protein 1 (MCP-1), and RANTES	(119, 132)
HSV-1	EMT-6 (permissive murine epithelial-like cell line)	ICP0, ICP4, ICP8, ICP27 independent	Viral replication dependent NF-κB. Increased levels of MyD116, GADD34	IL-6. IL-1, IL-7, IL-10 (2-12 h)	(60)
HSV-2	J774A.1 (murine macrophage cell line)		Viral replication dep. NF-κB	IL-12	(63)
HSV-1 HSV-2	Human PBMC Murine peritoneal cells, J774A.1, RAW264.7	ICP0, ICP4, ICP27 independent, dsRNA	PKR NF-κB p38 dependent	IL-6	(99)
HSV	Human vesicle fluid			High IL-1β, IL-6, MIP-1β moderate IL-1α, IL10, low IL-12 (day 1)	(89)
HSV-1	Vero	gH	JNK		(52)

Virus	Cell line	Viral gene	Signaling pathway	Induced cytokine	Reference
HSV-1 HSV-2	Human adult and newborn keratinocytes	72 h p.i.		IFN	(120)
U.V. HSV-1	Mice deficient IFNβ and IFNAR	2, 4, 6 h p.i.		IFN-α	(7)
U.V. HSV-1	Mice mononuclear leukocytes	3-9 h		IFN-α/β	(31)

Innate immunity and NF-κB

Overview. Innate immunity is the first line of the host defense system against invading pathogens. The main players in innate immunity are the phagocytes, such as macrophages, neutrophils, and dendritic cells. These cells recognize invading pathogens by germ-line-encoded pattern recognition receptors (PRRs), which are mainly dependent on the Toll-like receptors (TLRs) family. The engagement of TLRs with microbial products activates and initiates several intracellular signal transduction pathways, such as NF-κB, JNK (MAP Kinase) (152). The activation of NF-κB is one of the most prominent and best characterized signal transduction pathways. When TLRs bind with their ligands, TLRs subsequently recruit adapter MyD88 and interact with MyD88 via a homotypic TIR (Toll/IL1-receptor homologous region) connection. The death domain of MyD88 then recruits IRAK (IL1 receptor-associated kinase) to the receptor complex. IRAK is then autophosphorylated and dissociated from the receptor complex and recruits TRAF6

(TNF receptor associated factor 6), which in turn activates downstream kinases such as NIK or MEKK1. Activated NIK or MEKK1 is individually capable of activating the IκB kinase (IKK) complex. Ultimately, the IκBα subunit is phosphorylated and degraded, releasing NF-κB, leading to nuclear translocation of NF-κB and modulation of target genes transcription.

NF-κB regulates the expression of a variety of genes that play important roles in innate immunity. These NF-κB target genes include those encoding cytokines (e.g.IL-1β, TNF-α, TNF-β, IL-2, M-CSF, GM-CSF, G-CSF, IFN-β, IFN-γ, IL-6, proenkephalin, TGF-β2), chemokines (CCL2, CCL3, CCL5, CXCL1, CXCL2, CXCL3, CXCL8, CXCL10), adhesion molecules (ELAM-1, VCAM-1, ICAM-1), acute phase proteins (angiotensinogen, serum amyloid A precursor, complement factor B, complement factor C4, urokinase-type plasminogen activator), and inducible enzymes (NO-synthetase, cyclooxygenase-2, perforin) (147). Which of these genes is modulated is dependent on which components of NF-κB complex bind to specific genes or transcription promoter complexes.

NF-κB background. NF-κB was first described in 1986 as a nuclear factor necessary for immunoglobulin k light chain transcription in B cells (57). Since it was first observed, NF-κB has been found to play a role in immune responses, apoptosis, cell growth control and development.

The NF-κB protein family. All NF-κB proteins contain a conserved N-terminal region, called the Rel Homology Domain (RHD) (about 300 amino acids in length). The N-terminal part of the RHD contains the DNA-binding domain, whereas the dimerization domain is located in the C-terminal region of the RHD. Close to the C-terminal end of the RHD is the NLS (Nuclear Localization Signal), which is essential for the transport of active NF-κB complexes into the nucleus. NF-κB proteins can be grouped into two classes: one includes the p105 and p100, which are precursor proteins to p50 (NF-κB1) and p52 (NF-κB2) protein, respectively, and the other class includes Rel (c-Rel), v-Rel, RelA (p65) and RelB protein (147). Only RelA (p65), RelB and c-Rel contain potent Transactivation Domains (TDs) within sequences in RHD C-terminal domains. The TDs consist of abundant serine, acidic and hydrophobic amino acids that are essential for transactivation activity. In contrast, p50 and p52 do not possess TDs, and therefore cannot act as transcriptional activators by themselves. The molecular structure of NF-κB is composed of various combination of NF-κB family polypeptides in a homo- or hetero-dimer forms. The p50/p65 complex is present in essentially all cells, and is usually the most abundant dimeric complex. Some of dimeric forms like p65/p65 or Rel/Rel or p65/Rel are present at very low levels, but play important roles on specific promoters. The members of the Rel/NF-κB family can form almost all theoretically possible homo- and hetero-dimers, but complexes that have never been detected are RelB/RelB, RelB/RelA, and RelB/Rel. The various dimeric complexes differ in their preference for certain κB sites, transactivation potentials, kinetics of nuclear translocation and levels of

expression in tissue. The p50/p65 tends to be rapidly translocated to the nucleus in response to extracellular stimulations (147).

Nuclear/cytoplasmic status of NF-κB forms. NF-κB normally resides in the cytoplasm and its NH2-terminal region, RHD, is bound by an inhibitory protein complex IκB, an inhibitor of NF-kB. Phosphorylation of IκBα by IκB kinase (IKK) results in the release of NF-κB, which then moves into the nucleus where it binds to cis-acting κB sites (5'-GGRNNYYCC-3') (R: purine, Y: pyrimdine) in promoters and enhancers of over 100 regulatory genes involved in inflammatory and chemotaxic cytokines, stress responses, apoptosis, growth factors, and transcription factors (6). Although NF-κB generally interacts with IκB in the cytoplasm of most cell types and translocates to the nucleus upon stimulation with many stimuli (e.g., cytokines, mitogens, physical stress, oxidative stress, chemical agent, parasite, bacteria and bacterial products, viruses and viral products), the ubiquitous p50/p50, p52/p52 homodimer or p50/p52 localizes largely to nuclei in unstimulated cells. The role of this factor in constitutive-type transcription is unclear, but it may serve as a transcription repressor and protect some genes from modulation by other forms of NF-κB transactivating factors (147).

The IκB (inhibitor of kappa light polypeptide gene enhancer in B cells) protein family. In mammals, the IκB family proteins include IκBα, IκBβ, IκBγ, IκBε, IκBζ, BCL3 (B-cell CLL/lymphoma 3) and the precursor Rel-proteins, p100 and p105. All members of the family share a partially conserved domain that harbors between six to

eight ankyrin motifs, each 33-amino acids in length (14) (114). These ankyrin motifs interact with the nuclear localization signal (NLS) located at the end of the RHD domain of NF-κB to interfere with NF-κB nuclear translocation and inhibit DNA binding of REl/NF-κB complex (9) (53) (65). All of the IκB members act in the cytoplasm, except IκBζ and BCL3, which are thought to act in the nuclei. Various members of the IκB family target different NF-κB complexes, e.g, IκBα and IκBβ interact preferentially with p65/p50 and c-Rel/p50, IκBε binds only to p65 and c-Rel hetero-and homodimers (153) (165), IκBζ binds to p50 subunit of p65/p50 and p50/p50(171) and BCL-3 associates exclusively with p50/p50 or p52/p52 (115). Due to the functional difference of each IκB family member, the transcription factor NF-κB can be regulated differentially by IκB subunits. For instance, IκBα is rapidly degraded upon phosphorylation, which leads to an immediate, but transient activation of NF-κB; IκBβ is slowly degraded, but is associated with a more sustained activation of NF-κB (6); IκBε regulates late, transient activation of NF-κB complexes (165); IκBζ is not rapidly degraded, but stably accumulates in the nucleus where it inhibits NF-κB activity or plays a direct role in transcriptional activation (155) (169); BCL-3 can displace inhibitory p50/p50 from DNA and free Iκ binding sites for interaction with other NF-κB forms (50), whereas the ternary complex of BCL-3 and p52/52 induces the transcription of the NF-κB target gene (cyclin D1) (49) (164) (130).

IκBζ. Three IκBζ variants have been found to date, transcript variants 1, 2 and 3. (80) (171) (155). Both transcript variants 1 and 2 were cloned from murine macrophage-like cell line RAW264.7, and their protein products were found to be localized in the nucleus

where they preferentially bind to NF-κB p50. The longer form, transcript variant 1 or IκBζ (L) or MAIL-L, is predominantly induced upon LPS stimulation (170). The shorter form, transcript variant 2 or IκBζ (S) or MAIL-S differs in the 5' UTR and coding region compared to variant 1 (171). The resulting IκBζ (S) is 99 amino acids shorter at the N-terminus compared to IκBζ (L). The transcript variant 3 was cloned from human cell lines and lacked exon 2 (nt-193-268) (155). All three forms of IκBζ harbor 6 ankyrin-repeats in the carboxyl-terminal region, similar to what is observed in other IκB proteins. Interestingly, the amino-terminal regions of these molecules exhibit no significant homology to any other known proteins.

In contrast to the typical cytosolic and constitutively expressed IκB proteins, IκBζ is localized in the nucleus, where it binds preferentially to the NF-κB p50 subunit, rather than the p65 subunit, without prevention of their nuclear translocation. *In vitro* experiments have demonstrated that the carboxyl-terminal domain with the ankyrin-repeats blocks DNA binding of p65·p50 heterodimers as well as p50 homodimers (171). Overexpression of IκBζ, however, results in augmentation of IL-6 expression in response to LPS, but inhibits TNF-α expression (112). These data suggest that IκBζ is a key regulator of NF-κB and exhibits dual positive and negative functions that is target gene specific.

Mutual regulation of NF-κB and IκBζ. IκBζ is hardly detectable in unstimulated cells, but is strongly induced by LPS, IL1 or other pathogen components through Toll-like

receptor. NF-κB activation is essential, but not sufficient for the induction of IκBζ (171) (46) (169) (155). IκBζ induction requires both NF-κB activation and stimulus-specific stabilization of its mRNA (170).

Mutual regulation of NF-κB and IκBα. The activity of NF-κB is usually tightly regulated in normal cells. Upon activation of NF-κB, cytoplasmic IκBα is phosphorylated and degraded. IκBα mRNA and protein are also induced about 20 min after the initial signaling (19) (150) (142) (38). This feedback inhibition presumably is destined to restore the inhibited NF-κB state and to control the NF-κB regulated genes whose functions can be harmful to the cells.

Induced synthesis of NF-κB. The precursor proteins p105 and p100 are induced after cellular stimulation with agents that also activate NF-κB (17) (16). Induced expression of the NF-κB precursors presumably replenishes NF-κB in the cytoplasm after NF-κB is translocated into the nucleus. These precursors may also be critical for limiting activation and retaining any newly synthesized NF-κB protein like RelB or cRel in the cytoplasm, either which may be imported once IκB is degraded in response to stimuli. In addition to p100 and p105, Rel and RelB are also found to be transiently upregulated in response to stimuli that activate NF-κB(22) (134). The expression of RelA, however, is essentially constitutive and only small increases, if any, have been noted following various cellular stimulations (109). Induction of NF-κB subunits may be necessary to sustain nuclear NF-κB activity over longer periods of stimulations.

NF-κB signaling pathway: activation of NF-κB activity. NF-κB is activated to translocate into the nucleus in response to numerous stimuli that include cytokines (TNF-α, TNF-β, IL-1α, IL-1β, LIF, IFN-γ, M-CSF, GM-CSF), mitogens (antigen, allogeneic stimulation, lectins, phorbol esters, diacylglycerol), bacteria and bacterial products (LPS, toxins), viruses (CMV, HIV-1, HSV-1, HHV-6, Newcastle disease virus, Sendai virus, Adenovirus 5) and viral products (dsRNA, HTLV-1 Tax, HBV Hbx, EBV EBNA-2/LMP), parasite, physical stress (UV light, ionizing radiation), oxidative stress (Hydrogen peroxide, Butyl peroxide) and chemical agents (Ceramide, Calyculin A, Cycloheximide) (118) (94). Following exposure of a cell to an inducer, NF-κB-inducing kinase (NIK) or mitogen-activated protein kinase/ERK kinase kinase (MEKK) is activated and IκB kinase (IKK) complex is phosphorylated. IKK complex, which is comprised of phosphorylated IKKα, phosphorylated IKKβ catalytic subunits, and IKKγ regulatory subunit, targets IκB and results in IκB subunit phosphorylation (58). Phosphorylated IκB subunits are then recognized by the β-TrCP-containing SCF ubiquitin ligase complex, resulting in ubiquitination of the subunit and degradation by the 26S proteasome (141). The degradation of IκB subunits exposes the nuclear localization signal of the previously complexed NF-κB protein. NF-κB then translocates from cytoplasm to the nucleus where it binds to enhancers or promoters of target genes (148), unless it is retained in the cytoplasm by other mechanisms. In this canonical pathway, IKKβ, but not IKKα, is essential for inducible IκB subunit phosphorylation and subsequent degradation.

A second, evolutionarily conserved NF-κB pathway is strictly dependent on IKKα, but independent of IKKβ and IKKγ (143). In this pathway, TNF-cytokine family, but not TNF-α, activates NIK, which targets IKKα. Phosphorylated IKKα results in the phosphorylation of p100/RelB and subsequent p100 processing to p52 by an ubiquitinated-dependent cleavage. The p52/RelB dimer is then translocated into the nucleus. This alternative NK-κB activation pathway is only involved in adaptive immunity, which is required for B cell maturation and formation of secondary lymphoid organs. The classical NF-κB pathway, however, is mostly observed in innate immunity. Although it is clear that many different inducers initiate NF-κB signal transduction pathways as described above, several kinases, e.g., double-stranded RNA activated kinase (PKR) (81), Raf-1 protein kinase (88), PKCζ (42), and nuclear factor kappaB kinase (61), have also been implicated in the phosphorylation of IκB subunits, which is then followed by activation of NF-κB.

HSV-1 and NF-κB. It has been documented that HSV-1 activates NF-κB in C33 cells (human epithelial cells), U2-OS cells (human epithelial cells), and primary human fibroblasts (68) (122). NF-κB activation occurs in a biphasic manner (122) (59). The initial activation occurs very rapidly (within 30-45 min) and in a transient manner, and has been suggested to be related to a gD-receptor interaction (98). The second activation is delayed (within 3 and 4 hours), robust and sustained, and is dependent on virus entry and de novo ICP4 and ICP27 synthesis and turnover of IκBα and IκBβ (122) (2). The function for NF-κB activation by HSV-1 is to enhance the efficiency of virus replication

(122) and induce the expression of proinflammatory cytokines, interferons (87) (121) (99).

In summary, NF-κB has been shown to play as a central coordinator in the induction of a variety of genes critical for immune and inflammatory responses. Research has tended to focus on NF-κB signaling in the effector functions of hematopoietic components of the immune system, and its role has been well established in innate responses to all classes of pathogen. In addition, there is now increasing evidence to support a broader model in which NF-κB also supports the resolution of inflammatory responses, and initiation the coordination of the adaptive immune response. Yet, the exact molecular mechanism and interactions between upstream and downstream molecules in NF-κB signaling pathway can be pathogen- or cell type-specific. The characterization of the biochemical events leading to NF-κB activation is still a major challenge. However, understanding the molecular details involved in the regulation of NF-κB activity will allow us to modulate this pathway to control human diseases that originate by infections.

Part 2. Research Goals

B virus is one of the most feared occupational health hazards in the biomedical research community that depends on macaque monkeys or cells/tissues derived from these animals for research advances. Zoonotically infected humans can succumb to B virus infection within weeks to years post exposure, but infected macaques show little-to-no sign of infection. From observations made from human infections, it is known that B virus can invade the CNS very quickly. The infection tends to localize to the brainstem, causing disruption of autonomic functions including respiration, eventually resulting in death. During this time, the adaptive immunity against B virus is either not generated or not effective. Therefore, **we hypothesize that B virus infection in human cells is associated with defective induction of the innate immune response.** This hypothesis is also based on clinical observations of HSV-1 and HSV-2, as well as other herpesvirus infection outcomes in the presence and absence of innate immune responses. This hypothesis can be examined only partially since definitive, controlled infections in the humans can never be done. Studies proposed herein, however, will allow examination of early host responses that occur within target infected cells. To test the hypothesis, the following specific aims were designed:

1. develop a human cell culture model to identify early host responses to B virus infection;
2. identify virus-mediated human host cell responses at early time points of infection that target immune cell functions;
3. design and test blocking strategies to determine whether modulation of early events in the initially infected cell can alter immune response outcomes observed and described.

Experiments to accomplish each specific aim will distinguish infection-related events that may result in the observed outcomes of B virus infection in human hosts. Examination of the observed sequence of events in human cells will identify potential targets that can be utilized to modulate infection-related events in zoonotically infected humans.

SECTION II: MATERIALS AND METHODS

Cells and viruses

Monolayers of human foreskin fibroblast (HFF) (ATCC, CRL-2097) cells were grown in Eagle's minimum essential medium (Mediatech, Herndon, VA) supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (FBS). B virus laboratory strain E2490 and HSV-1 MacIntyre (ATCC, VR-539) were propagated in Vero cells (ATCC, CL81). All infections with B virus were performed under strict BSL-4 conditions in accordance with the Biosafety in Microbiological and Biomedical Laboratories (BMBL, 4th edition) manual (157). This laboratory is a registered facility for handling B virus under the Select Agent status.

Growth kinetics of viruses

The plating efficiency of viruses on Vero and HFFs were performed by 10-fold serial dilution of virus stocks and plated onto cells in 6-well plates. Virus titer was determined by counting plaque formation 2 days p.i. after crystal violet staining. To quantify the expression levels of viral genes, PCR primer sequences there were conserved in both B virus and HSV-1 were used to amply ICP0 (5'-CTCGAGCGTGGTGGCCAT-3' and 5'-GGGTCATCCAGAGGCCGT-3') and TK (5'-TGGACCTGGCCATGCTGTC and CAGCAGGTCGAGCGTCCAC-3') gene. The PCR cycle used was as followed: 95°C for 15 min, 35 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 10 min for one cycle. PCR products were quantified by running 1.5 % agarose gels after ethidium

bromide staining and analyzed by Kodak 1D Image analysis software. PCR band intensities were normalized to the band intensity of the control gene, β-actin.

Microarray and data analysis

Replicate cultures of HFF cells were mock-infected or infected with B virus (E2490) or HSV-1 (MacIntyre) at a multiplicity of infection (m.o.i.) of 10 at 4°C for 1 h, a time point designated as 1 h post infection (p.i.). Virus inoculum was removed after 1 h p.i., and the incubation temperature was then shifted to 37°C. Total cellular RNA isolated at 1, 3 and 5 h p.i. was used to generate cRNA for DNA microarray analysis using HG-U133 A/B gene chips (Affymetrix, Santa Clara, CA). Microarrays were performed according to manufacturer's instructions. Chip hybridization results were quantified using the GeneArray Scanner and GeneChip Operating Software 1.4 (Affymetrix, Santa Clara, CA). Microarray data was deposited into the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO) repository under the assigned accession number GSE4521.

Resulting microarray image files were analyzed using the R-Bioconducting package (http://www.bioconductor.org) analysis. The hybridization intensity of each probe was generated by running *affy* package, which processed an image of the microarray (stored in a .DAT file) to produce a .CEL file to contain signal intensity of each probe on the array. To normalize data, Robust Multichip Analysis (RMA) algorithm was used (15). Normalized data were analyzed by False Discovery Rate (FDR) (q cut-off value <0.002) and two-factor analysis of variance (ANOVA) (p cut-off value < 0.003).

Data were also imported into GeneSpring GX v7.2 (Silicon Genetics, Redwood City, CA) to perform hierarchical gene clustering (gene tree) across different time points post infection. To calculate the gene expression level, the average normalized expression level of a given gene per condition was compared to the mean of all normalized gene expression levels across all genes. Any genes having ratios greater or equal to 1.0 were defined as up-regulated genes; and ratios having less than 1.0 as down-regulated genes. Mock-infected samples were also compared for normalization of comparative data exploring differences between B virus and HSV-1.

Biological function and network analysis

A total of 1000 genes with greatest-fold changes that passed FDR, q cut-off values < 0.002 and ANOVA p cut-off values < 0.003 from each time point were analyzed for their biological functions and networks by the Ingenuity pathway analysis software (Ingenuity, Redwood city, CA). Global functional analysis was used to specify the significance of how likely it was that genes from the dataset file under investigation participate in that function. The significance for each function was expressed as the negative exponent of the Fischer's exact test p-value, which was calculated by comparing the number of user-specified genes of interest that participate in a given function relative to the total number of occurrences of these genes in all functional annotations stored in the Ingenuity Pathways Knowledge Base. For biological network generation, imported genes that were mapped to their corresponding gene objects in the Ingenuity pathways knowledge base, called focus genes, were overlaid onto a global molecular network. The

likelihood of the focus genes in a network being found together due to random chance was displayed as the score. For example, a score of 2 indicated that there was a 1 in 100 chance that the focus genes were found together in a network due to random chance. Therefore, scores of 2 or higher had at least a 99% confidence of not being generated by random chance alone. In this study, a score ≥ 45 was used as the cut-off value to select for highly significant biological networks regulated by B virus and HSV-1.

Real-time RT-PCR

Total RNA was extracted from mock- and virus-infected cells at 1, 3, and 5 h p.i. using QIAGEN's RNeasy Total RNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription reactions were performed on 2 μg of total RNA using an oligo dT primer (Promega, Madison, WI) with SuperScript II (GIBCO BRL, Carlsbad, CA). Real-time PCR reactions were performed in a 30µl volume that included dilute cDNA sample, primers (1µg/ml), probe and TaqMan Universal PCR Mater Mix (Applied Biosystems, Foster City, CA) by using Applied Biosystems Prism 7900-HT sequence detection systems. Primers and probes used were designed by Primer Express v1.5 (Applied Biosystems, Foster City, CA)**.** To compare mock- and virus-infected cell samples, relative changes in gene expression were determined by using the relative standard curve method for quantification according to the manufacturer's instructions. To calculate the fold-change ratio comparing virus infected to uninfected samples, genes having ratios greater or equal to 1.0 were defined as

up-regulated genes; and ratios having less than 1.0 as down-regulated genes. These fold changes were quantified more precisely with quantitative RT-PCR.

Isolation of neutrophils

Human blood was anticoagulated with 3.8% sodium citrate (use 6 ml of 3.8% sodium citrate for every 54 ml blood). Blood samples were centrifuged at 400 x g for 10 min at 4°C to remove platelet-rich plasma supernatant. The leukocyte-rich fraction containing red blood cells were separated by adding cold 2% dextran (in 0.9% sodium chloride) to total volume of 50 ml. Red blood cells was sedimented under gravity for 35 min at 4°C, and the leukocyte-containing top fraction was collected and centrifuged at 400 x g for 10 min at 4°C. The resulting leukocyte-enriched pellet was resuspended in 10 ml of cold 0.9% NaCl and then layered over onto the top of 3 ml of cold ficoll-paque (GE Healthcare Bioscience AB, Piscataway, NJ). After centrifugation at 400 x g for 30 min at 4°C, neutrophils were found in the pellet. The neutrophil-rich pellets were collected; contaminating red blood cells were removed by adding cold hypotonic lysis buffer (155 mM NH_4Cl, 2 mM $NaHCO_3$, 0.1 mM EDTA). Neutrophils were then washed once with cold Hanks balanced salt solution (HBSS) without calcium and magnesium. Cells were resuspended at a density of $5x10^7$ cells/ml in cold HBSS and remained viable suspended in HBSS for about 4 hours at 4°C.

Neutrophil transmigration assay

HFF cells prepared in T-150 flasks were either mock infected or exposed to B virus or HSV-1 at m.o.i. of 10. At time points 1, 2, 3, 4, 5 and 6 h p.i., collected culture fluids were centrifuged at 400 x g for 10 min and the supernatants were frozen at -80°C for subsequent assays.

Neutrophil transmigration assays were performed by adding freshly isolated PMN (1×10^6 cells in 150 μl HBSS) to the upper chamber of the transwell setups separated from the bottom chamber by a collagen coated 5 μm pore size polycarbonate filter with a surface area of 0.33 cm^2 (Costar, Cambridge, MA). The transwells were coated with collagen (purified from rat-tails, 50ug/ml in 70% ethanol) and dried in a hood overnight before use. On the bottom chamber, 0.5 ml of tested samples contained (1) fresh medium (negative control) (2) medium plus 0.1μM fMet-Leu-Phe or 0.4μg/ml IL-8 (positive control) (3) supernatants from mock-infected cells (4) supernatants from HSV-1 infected cells were added in triplicate wells. The assembled chambers were incubated at 37 °C for 1 h to 3 h and neutrophils migrating into the lower chamber were quantified by solubilizing neutrophils with 1% Triton X-100 and assaying for myeloperoxidase (MPO) activity by measuring optical density units (OD) at A_{405} after adding substrate ABTS (2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid). The percentage of cells transmigrating across the transwell filter was calculated by a standard curve method, which was constructed by serial diluting cell numbers (10-fold serial dilution of 1×10^6 cells) versus their OD_{405} values. Student's t-test was used to compare the chemotactic

activity between supernatants from virus- and mock-infected cells and a p value of <0.05 was considered to be a significant change.

Virus infected cell supernatant-mediated inhibition of neutrophil transmigration

Neutrophils were isolated as described above. In the transwell setups, $1x10^6$ neutrophils were added to the upper chamber. The neutrophil transmigration inducers, 0.2 μM f-MLP or 200 ng/ml IL-8, were added into the lower chamber of the transwells setups in addition to the presence of 0.5 ml tested mocked-, or B virus- or HSV-1-infected cell supernatants. Neutrophil transmigration was initiated by incubation at 37°C and neutrophil migration into the f-MLP or IL-8-containing lower chambers was quantified by MPO activity measurement as described above.

ELISA (enzyme-linked immunosorbent assay) for detection of IL-8

HFF cells prepared in T-150 flasks were either mock infected or infected with B virus or HSV-1 at m.o.i. of 10 at 4°C for 1 hour. This time point was designated as 1 h post infection. Inocula were then replaced with fresh medium and cells were incubated at 37°C/5%CO_2. At 2, 3, 4, 5 and 6 h time points p.i., culture fluids were collected and stored at -80°C for the subsequent IL-8 ELISA assay (RDI, Concord, MA) according to the manufacturer's instructions.

Quantification of NF-κB p50 nuclear translocation

HFF cells plated into 96 well microplates (Costar, Coming, NY) at $2x10^4$ cells per well were incubated with B virus at m.o.i. of 10 for 1, 2, 3, 4, 5, and 6 h p.i. HFF cells treated with TNF-α (0.1μg/ml) for 1 h were used as a positive control. Cells were fixed in 4% formaldehyde for 15 min after infection, permeabilized in 0.2% Triton X-100 for 10 min, and then washed with phosphate buffer saline (PBS). The nuclei were stained with Hoechst 33342 (Molecular probes Inc., Eugene, OR) for 15 min at room temperature. After washing slides with PBS three times, slides were treated with blocking solution (1% bovine serum albumin in PBS) at 37°C for 1 h. Immunofluorescent staining was performed by incubating slides with rabbit anti-NF-κB p50 (1:100) antibody at room temperature for 1 h, followed by incubation with fluorescein conjugated goat anti-rabbit IgG (1:200). Cells were then washed, sealed and stored at 4°C until they were scanned on an ArrayScan HCS Reader (Cellomics, Pittsburgh, PA), with the Cytoplasm to Nucleus Translocation BioApplication, using a 20x objective. The activation of NF-κB p50 was analyzed by measurement of the amounts of NF-κB p50 translocation from the cytoplasm to nucleus and represented as the difference in average fluorescent staining intensity between the nucleus and cytoplasm.

Preparation of nuclear extracts

HFF cells in T-150 flasks were mock-infected or exposed to B virus or HSV-1 at m.o.i. of 10 and harvested at 1, 2, 3, 4, 5, and 6 h p.i. Before harvesting cells, cells were washed twice with cold PBS. Cells were scraped into 10 ml cold PBS and centrifuged at

1500 x g for 5 min. Cell pellets were suspended in 10 ml of hypotonic 0.1 M Tris-Cl, 0.01 M NaCl, and 0.0015 M $MgCl_2$ (TSM) and stored on ice for 30 min. Swollen cells were homogenized with 100 strokes in a cold Teflon: glass Potter-Elvehjem homogenizer. The homogenate was then mixed with 1ml of Triton X-100 (20% w/w) to a final concentration of 1.8%. Nuclei, recovered by centrifugation at 1000 x g for 10 min, were suspended in 50 μl buffer C (20 mM HEPES, pH 7.9, 0.4 N NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), and the tube was vigorously rocked at 4°C for 25 min on a shaking platform. The nuclear extracts were centrifuged at 20,000 x g for 5 min at 4°C and the supernatant was frozen in aliquots at –70°C. Protein concentrations were determined by Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL). All further manipulations of inactivated B virus infected materials were done in BSL-3.

NF-κB p50 DNA binding activity assay

NF-κB p50 DNA binding activity assay was performed by an ELISA-based TransBinding NF-κB p50 Assay Kit (Panomics, Redwood City, CA) according to manufacturer's instructions. In this kit, an oligonucleotide containing a NF-κB consensus binding site was immobilized on the 96-well plate. Activated NF-κB p50 from 10 μg nuclear extracts specifically bound to this oligonucleotide was detected by antibody directed against the p50 subunit. An additional secondary horseradish peroxidase (HRP)-conjugated antibody provided a colorimetric optical density readout by spectrophotometry (A_{450}).

Immunofluorescent staining

HFF cells in 16-well chamber slides were infected with B virus or HSV-1 at m.o.i. of 10 for 1, 2, 3, 4, 5 and 6 h. Cells were fixed in 3.7% formaldehyde for 15 min after infection, permeabilized in 0.2% Triton X-100 for 10 min, and then washed with phosphate buffer saline (PBS). Nuclei were stained with Hoechst 33342 (Molecular probes Inc. Eugene, OR) and F-actin was stained with Alexa Fluor 568 phalloidin (Molecular probes Inc. Eugene, OR). Cells were then stained with the primary antibodies rabbit anti-IκBζ IgG (Imgenex, San Diego, CA) and rabbit anti-NFκB p50 IgG (Santa Cruz Biotechnology Inc. Santa Cruz, CA) (H-119) at room temperature for 1 hour, followed by incubation with fluorescein conjugated secondary antibody goat anti-rabbit IgG. Slides were mounted by VECTASHIELD (Vector Laboratories) and were observed with a Zeiss LSM 510 confocal laser scanning microscopy.

Knockdown IκBζ by siRNA

The siRNA duplexes, MAIL siGENOME SMARTpool, against IκBζ (GeneBank no. NM_001005474, NM_031419) was purchased from Dharmacon (Lafayette, CO). Silencer Negative control #1 siRNA was purchased from Ambion (Austin, TX). HFF cells ($3x10^5$) (ATCC-CCL110) were washed and resuspended in 150 μl siRNA electroporation buffer (Ambion, Austin, TX). In a 1-mm gap cuvette, 150 μl of cells were mixed with 100 pmol siRNA and electroporated four times at 150V, 400 μs, 10 s between pulses. Subsequently, cells were placed at 37°C for 10 min and then transferred to 24-well plates with 500 μl pre-warmed medium per well. After incubation at 37°C/5%CO_2

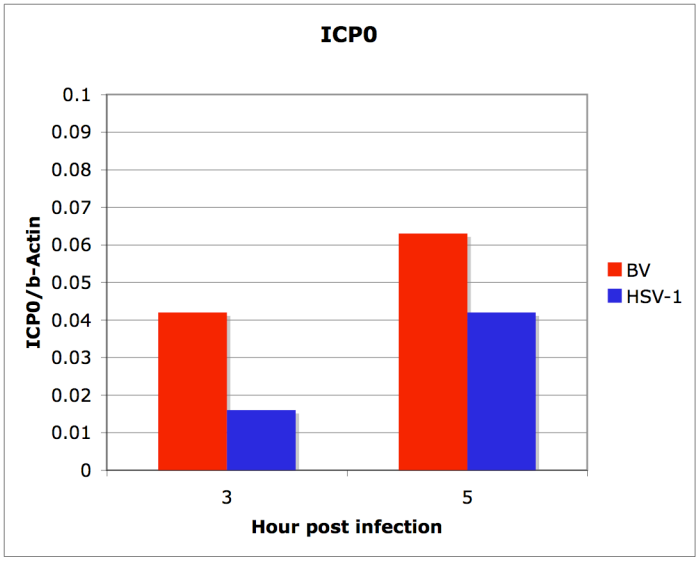
for 72 hours, siRNA-treated cells were mocked infected or infected with B virus at m.o.i. 10. Total RNA was at 1, 2, 3, 4, 5 and 6 h p.i. using TRI reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's instructions. Real-time RT-PCR of specific NF-κB target genes (e.g. IL-6, IL-8, CXCL1, CXCL2, CXCL3, CCL2) was performed by using Prism 7900-HT sequence detection systems (Applied Biosystems, Foster City, CA). To determine IκBζ gene knock down rate, relative changes in IκBζ gene expression of siRNA-treated samples v.s. mocked infected cells were determined by using the relative standard curve method for quantification according to the manufacturer's instructions. To compare cytokine gene expression between B virus infected IκBζ knockdown cells versus negative control siRNA-treated B virus infected cells, relative changes in gene expression were determined by using the relative standard curve method for quantification as well.

SECTION III. RESULTS

Part 1. Development of a human cell culture model system to investigate the early host responses to B virus infection.

B virus and HSV-1 growth kinetics in human foreskin fibroblasts. To simulate the initial encounter experienced by B virus at the onset of a zoonotic infection, human foreskin fibroblasts grown in culture were chosen as the *in vitro* cell culture model, because they are likely to be one type of target cells infected *in vivo*. They have been shown to be productively infected, and produce HSV-1 viral proteins in vitro (126). B virus and HSV-1 stock propagated from Vero cells, maintained between passages 130-140, were titrated on both Vero and HFF cells to compare the plating efficiency on different cell lines. This was used to establish that the replication kinetics and virus replication were equivalent over the same time periods. In essence, this technique was used as an outcome measurement that reflected the growth curve of these viruses in two different cell lines. B virus has a ~4 fold reduction of plating efficiency on HFFs related to that on Vero, whereas the plating efficiency of HSV-1 on both Vero and HFFs has no significant difference. The growth kinetics of B virus was slightly faster by 4-6 hours post infection than HSV-1 on HFFs, because the expression levels of immediate early (α) gene ICP0 and early (β) gene thymidine kinase (TK) in B virus infected cells at 3 h and 5 h p.i. were higher than that observed in HSV-1 infected cells (Fig 1). Molecular analysis

a



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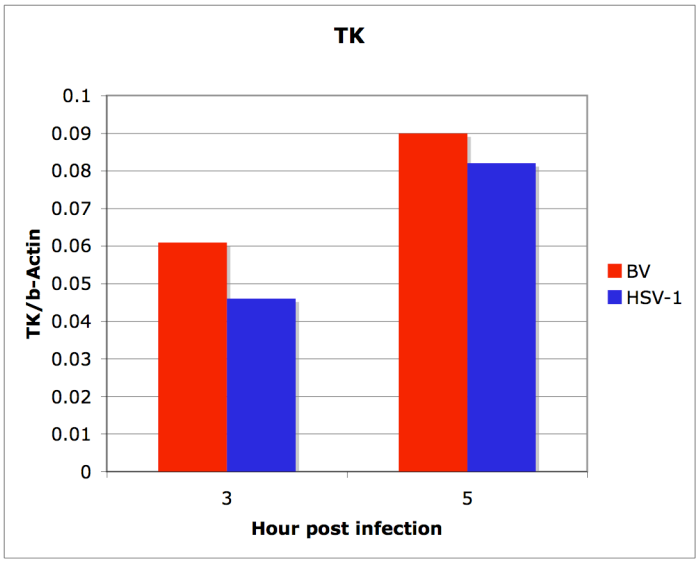


Fig 1. Expression levels of ICP0 (a) and TK gene (b) from B virus and HSV-1 infected HFFs. Quantification of the PCR-amplified viral gene products was performed by densitometric analysis by using Kodak 1D Image analysis software. PCR band intensities were normalized to the band intensity of the control gene, β-actin.

of the kinetics of replication using measurement of α, β, and γ classes of proteins that characterize alphaherpesviruses revealed the differences in replication kinetics. Collectively, these data provided a complete picture of the virus growth curves and formed the basis for subsequent infections to establish that identical virus loads were used in the comparisons of B virus and HSV-1 in HFF cells. All HFF cells used in the subsequent experiments were derived from the same donor cells, which were expanded from the source cells provided by ATCC.

Microarray experimental design. The early host cellular events induced by these two closely related simplexviruses, B virus and HSV-1, in a common cell type, human foreskin fibroblasts, were comparatively analyzed in microarray experiments using the Affymetrix human cDNA microarray U133 set to obtain a global snapshot of virus-induced host cell gene activities at different time points post infection. Total cellular RNA isolated from duplicate infected samples at 1, 3 and 5 h p.i. was prepared for microarray analysis.

To monitor microarray data quality, Affymetrix™ provides several controls to check variability associated with mRNA extraction and purification, cDNA synthesis, labeling and purification, and signal detection. These controls include B2 Oligo hybridization performance on the boundaries of each gene chip, the average background and noise values, Poly-A controls (*B. subtilis* genes *dap*, *lys, phe, thr*, and *trp*) to check the entire target labeling process, hybridization controls to evaluate sample hybridization efficiency (*E. Coli* genes *BioB, bioC, bioD* and P1 bacteriophage gene '*cre'*), the internal

control genes (β-actin and GAPDH) to assess RNA sample and assay quality, and the number of probe sets called "present" relative to the total number of probe sets on the array. All of chips used for these studies passed all control criteria a requisite before data were accepted for analyses.

Although Affymetrix Data Mining Tool™ (DMT) can filter genes of interest based on the thresholds of certain quantitative and qualitative parameters, it is not a powerful tool in terms of data normalization and statistical analysis, both which were deemed to be important in this comparative analysis. Obvious differences in sensitivity and specificity have been substantiated among the different analysis methods. As a result, Robust Multichip Average™ (RMA) methods were implemented in the Bioconducting microarray analysis software to perform image-processing and data normalization, since RMA provides more accurate measurements of gene expression levels on gene chips (74) (1). These analyses were done in collaboration with Emory University's microarray facility, BIMCORE. The essential differences between RMA and the other methods are that RMA uses log-transformed perfect-matched probe signals on gene chips; it carries out a global background adjustment and across-array normalization using a formula that is based on a normal distribution; and it uses a linear model to estimate expression values on a log scale.

To identify a list of genes with statistically significant changes, stringent selection criteria were used by analysis of variance analysis (ANOVA, p-value) and false-

discovery rate (FDR or q-value) analyzing these studies. Fold change is a popular method to evaluate if a gene is differentially expressed. The drawback is that it is considered to be an inadequate statistical test, because it does not offer variance and confidence of the analyzed data (104) (21), both important points essential to robust interpretation of the experiments presented in this dissertation. ANOVA is a simple and effective alternative method to fold-change method. It estimates the probability (p value) that a difference in expression could have been observed by chance. To establish p value thresholds better, the emerging consensus in the microarray data analysis field (1) suggests that FDR (10), which is the fraction of false positives within a list of genes exceeding a given statistical cutoff, is more practical for dealing with the testing of multiple hypotheses within a single study in the microarray experiments. These statistical methods allow operator control of errors in a carefully designed studies.

Kinetic analysis of cellular gene expression after B virus and HSV-1 infection of HFF cells. To define early cellular signaling during B virus and HSV-1 infection in HFF cells, expression patterns of cellular genes during a 5 h period after virus inoculation were explored using human gene microarrays. Figure 2 provides an overview of the cellular gene expression patterns from B virus and HSV-1 during the first 5 hours of infection. In the first hour of incubation at 4°C, viruses were generally assumed to be only attached to cells, but had not penetrated into the cells (71) (95) because the growth properties of B virus resemble typical herpesvirus-characteristic replication (139) (67)

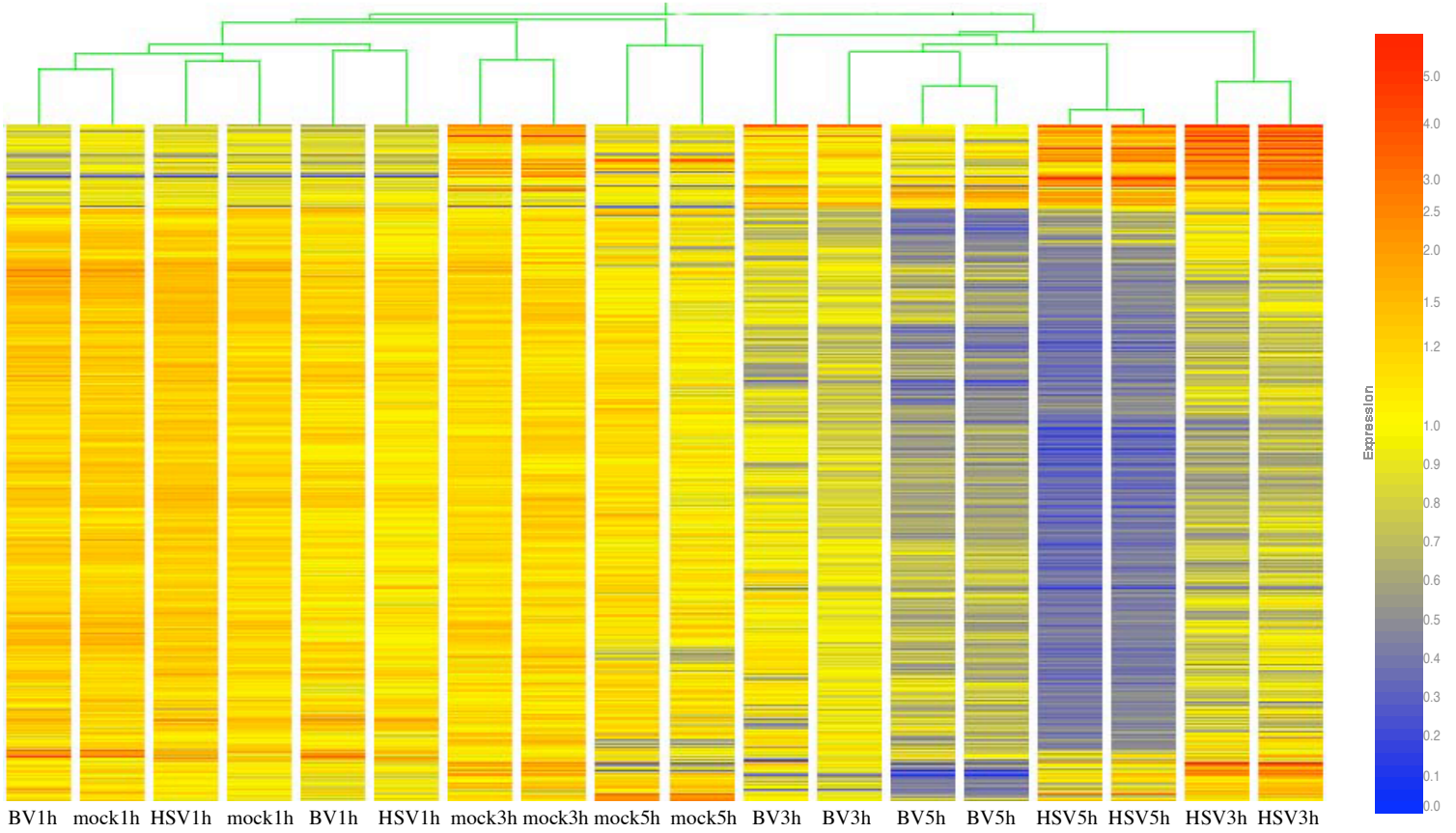


Fig 2: Hierarchical cluster analysis of DNA microarray data obtained from B virus and HSV-1 infected cells post 1 h, 3 h, and 5 h infection in HG-U133 set chips. Each square in the heat map represents the average normalized expression level of a given gene per condition. Red represents increased expression and blue represents decreased expression compared to the mean of all normalized gene expression levels across all gene chips. Samples with similar gene expression profiles are clustered together. The color bar represents normalized expression scale.

(166). The gene expression patterns were not observed to change significantly in any 1 h p.i. samples with the exception of ne gene, IL-6ST (interleukin 6 signal transducer, gp130), which was induced by both B virus (2.0-fold) and HSV-1 (2.23-fold). Further correlation analysis by R-square test (data not shown) also supported that the host cell gene expression levels in each of the B virus, HSV-1, and mock-infected samples at 1 h p.i. correlated precisely. In part, this reflects the lack of metabolic activity due to the 4°C temperature, and this serves as the starting point from which all cells, infected and uninfected were released for subsequent measurements at 37°C in the subsequent experimental time points post infection.

After incubation temperature was shifted to 37 °C for another 2 h (3 h p.i.) and 4 h (5 h p.i.), host cell gene expression levels showed significant changes. More down-regulated genes were observed than the numbers of up-regulated genes in each of virus infected cells. Interestingly, there were fewer genes that were up-regulated or down-regulated in B virus infected samples than in HSV-1 infected samples at both 3 h and 5 h p.i.

B virus-infected cells was found to have 16 up-regulated genes at 3 h p.i., while HSV-1 was found to have 136 up-regulated genes with at least 2-fold increase (Fig 3). Three genes were commonly up-regulated by both B virus and HSV-1 include *Homo sapiens* mRNA for Nef-attachable protein, nuclear factor of kappa light polypeptide gene

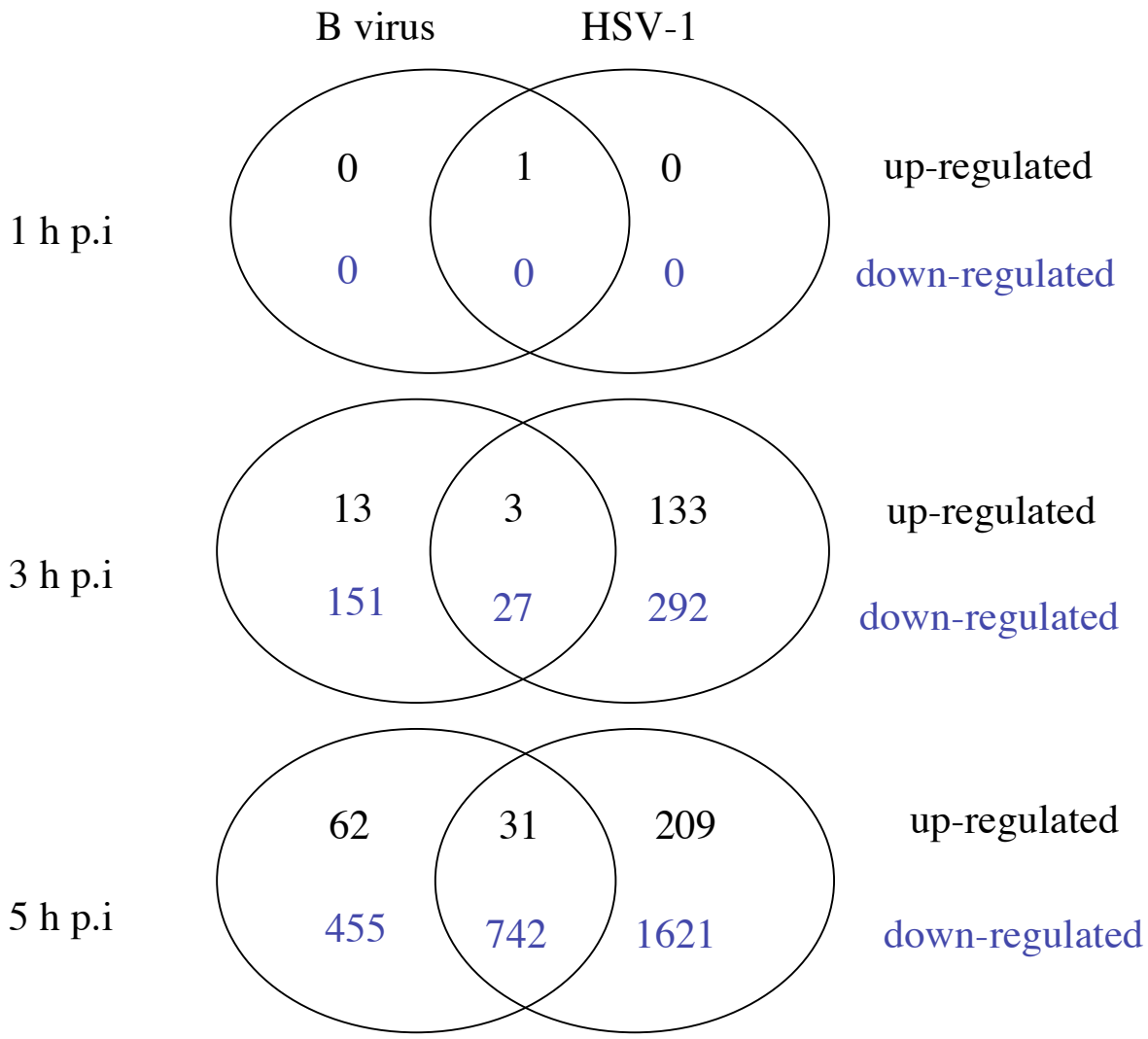


Fig 3: Venn diagrams of differential and overlapping regulated host cell gene numbers between B virus and HSV-1 infected samples at 1, 3, and 5 h p.i.

enhancer in B-cells inhibitor zeta (NFKBIZ), and splicing factor proline/glutamine rich (SFPQ). In B virus infected cells, 178 genes were found to be down-regulated; whereas for HSV-1, 319 genes were down-regulated with 27 commonly regulated genes between B virus and HSV-1 groups.

At 5 h p.i., the number of down-regulated host genes was greater than the number of up-regulated cellular genes. Data sorted with at least 2-fold change revealed that there were 93 genes up-regulated and 1197 genes down-regulated in B virus infected cells; whereas there were 240 genes up-regulated and 2363 genes down-regulated in HSV-1 infected cells. Between B virus and HSV-1 infected cells, there were 31 commonly up-regulated genes and 742 commonly down-regulated genes. Among the 31 commonly up-regulated genes, six genes were transcribed sequences with unknown functions, and the others included early growth response 1, cytoplasmic polyadenylation element binding protein 2, zinc finger SWIM domain containing 6, PRO1073 protein, *Homo sapiens* mRNA for Nef-attachable protein, UDP-glucose ceramide glucosyltransferase, *Homo sapiens* soggy-1 gene, SH3 domain and tetratricopeptide repeats 1, Down syndrome critical region gene, wingless-type MMTV integration site family member 1, POU domain, class 3 transcription factor 1, paired-like homeodomain transcription factor 3, transcription factor 8 (represses interleukin 2 expression), bridging integrator 3 and protease serine 22.

Biological functions of up-regulated genes in B virus and HSV-1 cells. Genes that were down-regulated were further sorted separately according to their q-values ($q<0.002$, and p-values ($p<0.003$). A total of 1000 genes with greatest fold-changes that passed q and p cut-off values from each time point were chosen for analysis of their biological functions. Ingenuity™, pathway analysis software, was used for this purpose. In B virus infected cells, after 3 h p.i. (virus attachment at 4°C for 1 hour to preclude virus penetration, followed by 37°C incubation to allow penetration for 2 h), the top 1000 up-regulated genes that mapped to the Ingenuity™ database can be classified into lipid metabolism, molecular transport, small molecule biochemistry, drug metabolism, gene expression, and other functions. HSV-1 induced a different set of genes relative to B virus gene expression, e.g., cancer genes, cell cycle, neurological disease, cellular growth, and proliferation functions, etc. To examine the significance of a given function of the virus induced genes at 3 h p.i., the five most significant functional groups induced by B virus were selected for comparison with HSV-1 and the results are shown in Fig 4a.

At 5 h p.i. (virus attachment at 4°C for 1 h followed by 37°C incubation for 4 h), the majority of genes expressed was decreased in B virus and HSV-1 infected cells. Those genes that were found to have increased expression in B virus infected cells belonged to the functional group of cell-to-cell signaling and interaction, renal and urological system development, cellular development, nervous system development and function, and tissue morphology. HSV-1 induced genes can be classified into cell-to-cell signaling and interaction, cancer, cell death, neurological disease and cell cycle (Fig 4b).

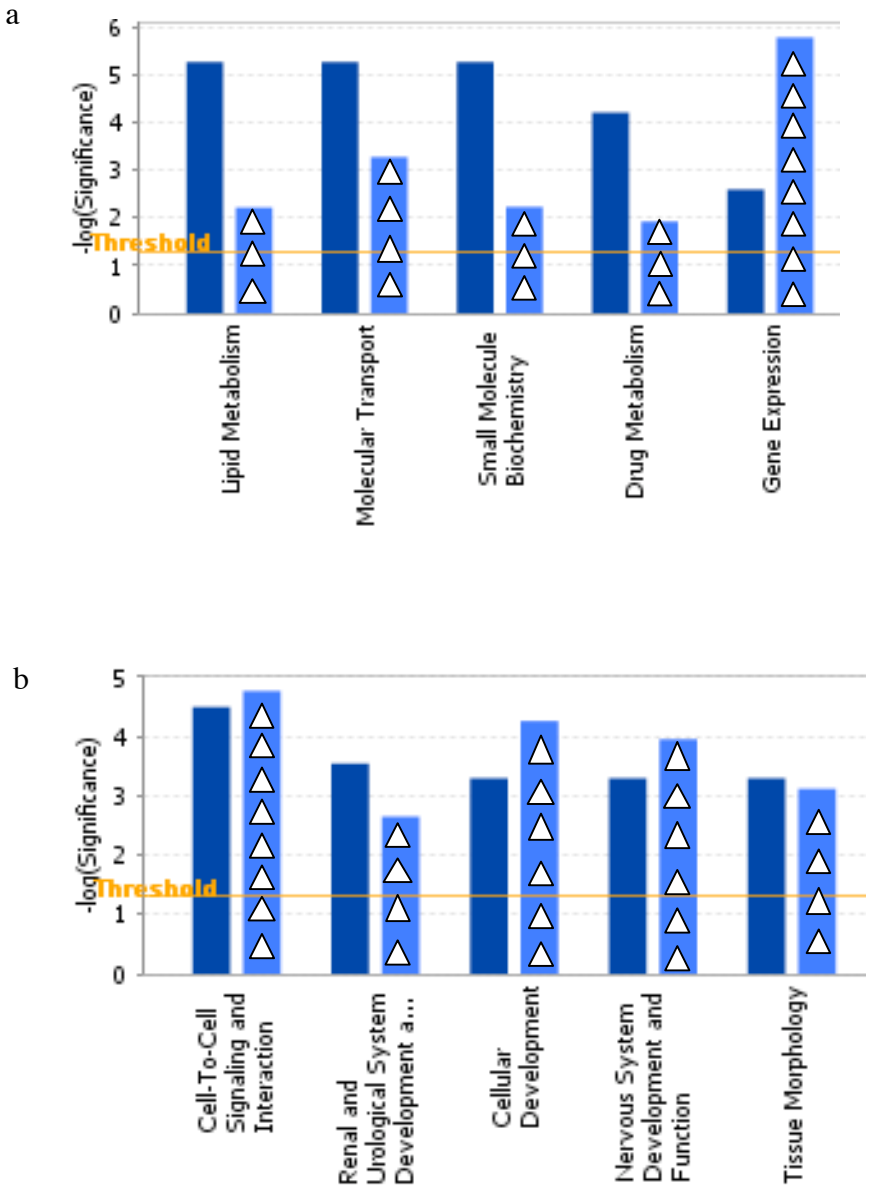
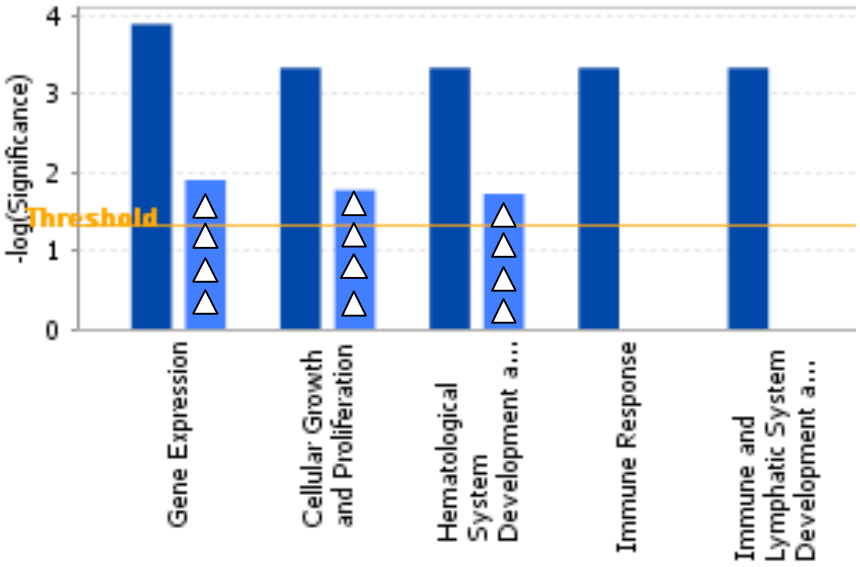


Fig 4: Comparison of B virus (dark blue) and HSV-1 up-regulated genes functions (light blue with triangles) at 3 h (a) and 5 h (b) p.i. Functions are listed from most significant to least and the orange horizontal line denotes the cutoff for significance (equal to a threshold of 1:20, i.e. a 1 in 20 chance that the listed functions were associated with the genes from your dataset by random chance alone).

Biological functions of down-regulated genes in B virus and HSV-1 cells. The top 1000 down-regulated genes with greater fold changes that passed q-values ($q<0.002$) and p-values ($p<0.003$) in B virus infected cells at 3 h p.i. were grouped into gene expression, cellular growth and proliferation, hematological system development and function, immune and lymphatic system development, and immune response. The down-regulated genes in HSV-1 infected cells at the same time point were involved in the embryonic development, tissue development, organismal development, auditory disease and cell morphology. The comparison of gene functions that were down-regulated between B virus and HSV-1 is shown in Fig 5a.

The down-regulated genes in B virus infected cells at 5 h p.i. were related to the function of embryonic development, tissue development, cardiovascular system development and function, cellular growth and proliferation, and cellular assembly and organization, etc. In HSV-1 infected cells at 5 h p.i., the down-regulated genes' functions were found in cancer, gastrointestinal disease, genetic disorder, cardiovascular disease and cell morphology (Fig 5b).

a



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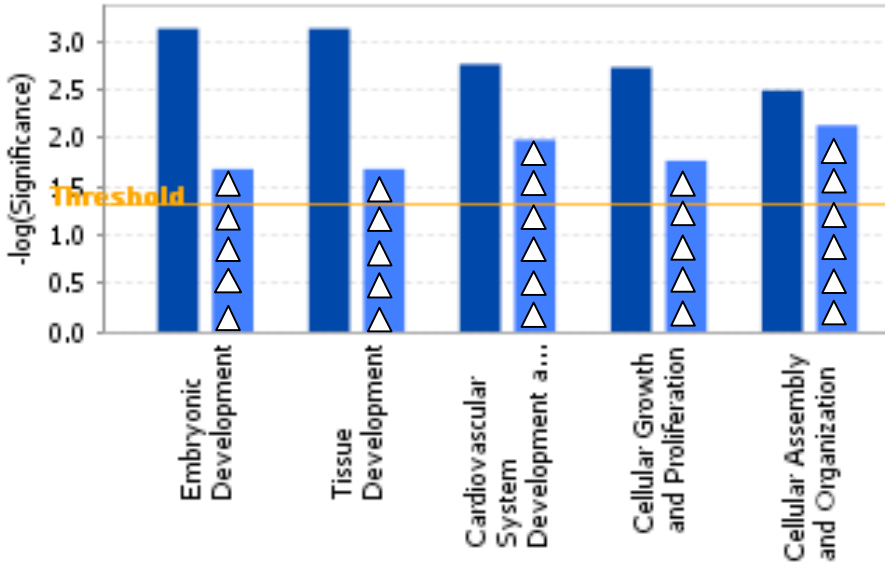

Fig 5: Comparison of B virus (dark blue) and HSV-1 down-regulated genes functions (light blue with triangles) at 3 h (a) and 5 h (b) p. i. Functions are listed from most significant to least and the orange horizontal line denotes the cutoff for significance (equal to a threshold of 1:20, i.e. a 1 in 20 chance that the listed functions were associated with the genes from your dataset by random chance alone).

Biological network affected by B virus and HSV-1 infection. To explore molecular relationships between the observed transcripts that had statistically significant changes ($q<0.002$, $p<0.003$), networks associated with biological functions were generated by Ingenuity™ pathway analysis tool, and the results are shown in Table 2 & 3. Five networks within up-regulated genes were found in HSV-1 at 3 h p.i., and these were involved in inflammatory disease, cell-to-cell signaling and interaction, hematological system development, cell death, gene expression, cancer, cell morphology, cellular movement, cellular development, cellular comprise, and dermatological diseases and conditions. Interestingly, the first network that has been observed in HSV-1-infected cells regulated many cytokines that were related to the NF-κB pathway. Similar functional networks were also found in B virus infected cells, but the expression levels of these up-regulated genes that composed of this network were all less than a 2-fold change, except for NFKBIZ. At 5 h p.i. analysis of B virus infected cells revealed three networks within the up-regulated genes and were found to be related to cellular growth and proliferation, cellular development, tissue development, cardiovascular disease, organismal injury and abnormalities, tissue morphology, and hematological system development and function. In HSV-1 infected cells, 5 major networks were found within the up-regulated genes, associated with cell-to cell signaling interaction, hematological system development and function, cellular movement, cellular growth and proliferation, gene expression, cell death, reproductive system disease, cellular development, cancer.

Table 2. Biological network affected by B virus and HSV-1 infection at 3 h p.i. Ingenuity Pathways Analysis computes a score for each network according to the fit of that network to the user-defined set of Focus Genes. The score indicates the likelihood of the Focus Genes in a network being found together due to random chance. A score of 2 indicates that there is a 1 in 100 chance that the Focus Genes are together in a network due to random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone.

	Net	Genes	Score	Top Functions
B virus 3 h	1	AIF1, BCL3, CABP1, CCL24, CCRN4L, CP, CPB2, CYP2E1, DUSP5, F12, GAD1, GAD2, GAS6, HGFAC, IL1B, IL1RL1, ITGAX, LCN2, LYL1, MARCKSL1, MUC5AC, NFKB1, NFKB2, NFKBIA, NFKBIE, NFKBIL2, NFKBIZ, NRAP, PBEF1, PIM3, RELB, TNFAIP2, TNFAIP3, YY1, ZA20D2	46	Gene Expression, Dermatological Disease and Conditions, Inflammatory Disease
	2	ADRB3, ARID3A, BTK, CD19, CHGA, CHKA, CSF3R, DNM2, EMR1, ERG, FCERG, GAS, GNA01, GRIN1, GRIN2A, GRIN2D, HNRPD, IRF8, JUND, KIAA1893, KLF16, NPFF, PCBP2, PDPK1, PRKCE, PTPN5, RCOR1, RUNX1, SAP30, SP3, SPI1, SPIB, SRC, SYNGAP1, TCF7L2	46	Cellular Development, Immune and Lymphatic System Development and Function, Hematological System Development and Function
	3	APOA1, APOE, ARID1A, ATF3, C1QL1, CETP, CHES1, CRSP7, DYRK1A, ESR1, FBP1, GADD45A, HNF4A, IL1RN, ING1, LBP, LIPA, MAPT, MARK4, NCOR1, NFATC3, PNRC1, POMC, PRLH, RANBP9, SFTPB, SFTPC, SLC19A1, SMARCA4, SMARCD1, STK11, TFAP2C, TITF1, WNT1, ZNF335	46	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
HSV 3 h	1	BCL3, BDKRB1, CCL2, CCL20, CCRN4L, CD83, CXCL2, CXCL3, CXCL6, DUSP5, ECGF1, ELF3, F3, G0S2, GCH1, GDF15, IER3, IL1B, IL6R, KLF10, MARCKSL1, MAZ, MT1E, NFKB1, NFKBIE, NFKBIZ, PBEF1, PHLDA1, PIM3, PLK3, PTGES, PTX3, REL, SPHK1, TNFAIP2	46	Inflammatory Disease, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
	2	ACOX1, ALCAM, ARID4A, ATF3, BCL2L11, BTG2, C5orf13, DUSP7, EGR1, EGR4, EPAS1, ERN1, FOSL1, GADD45A, GADD45B, GAL, IL6, IL1RL1, ING1, JUNB, KLF5, LDLR, MCL1, NAB1, PDGFA, PPP1R15A, PRDM1, SIRT1, SOD2, STATIP1, STK11, TWIST1, WNT1, XBP1, ZFP36	46	Cell Death, Death Expression, Cancer
	3	ADM, BRD2, CALD1, CEBPD, CKS2, DLX2, DUSP6, EIF4A1, FTH1, GCN5L2, GTF2A2, ID2, KLF2, LIF, METAP2, MNT, MSX1, MSX2, MXD1, MYC, NGFB, NSEP1, p44S10, RELB, RPL10, RPL13, RPS6, RPS16, SP3, TAF4, TAF10, TNC, UBE2S, YAF2, YY1	46	Gene Expression, Cancer, Cell Morphology
	4	BDKRB2, COMP, CTSG, CYR61, DDX39, EDN1, ELL, EREG, FOS, FOSB, GAS5, GLCCI1, HBEGF, ICAM1, IL8, ITGB2, KLF6, MAP3K8, MAP3K14, NFATC1, NFKB2, NFKBIA, NR3C1, NTF3, PLAU, PLAUR, PPP2R2A, PTGS2, PVALB, SERPINB2, SERPINE2, TNFAIP8, TPM4, TSC22D3, VEGF	46	Cell Death, Cellular Movement, Cell-To-Cell Signaling and Interaction
	5	ABI1, ARHGEF2, BCL6, BCOR, BYSL, CCL7, CHKA, CXCL1, DUSP1, EGFR, EGR2, ETS2, GTPBP1, HOXA3, HOXA9, HOXC8, HOXD10, IER2, JUN, KRT18, MAFB, MAFF, MAFG, MAP2K3, MAP3K1, MAPK8IP3, MLL, PKNOX1, RBBP6, RPL4, SDC4, SNAI1, SOS2, TGIF, WSB1	46	Cellular Development, Cellular Compromise, Dermatological Disease and Conditions

Table 3. Biological network affected by B virus and HSV-1 infection at 5 h p.i. Ingenuity Pathways Analysis computes a score for each network according to the fit of that network to the user-defined set of Focus Genes. The score indicates the likelihood of the Focus Genes in a network being found together due to random chance. A score of 2 indicates that there is a 1 in 100 chance that the Focus Genes are together in a network due to random chance. Therefore, scores of 2 or higher have at least a 99% confidence of not being generated by random chance alone.

	Net		Score	Top Functions
B virus 5h	1	ACVR1B, ASCL1, TOH1, BHLHB3, BMP7, CCL11, CCL14, EPAS1, GAD1, GAL3ST1, HAND1, HES1, HES5, IGSF1, INHA, KIAA1036, LYL1, MATK, MEF2C, MEF2D, NFATC3, NODAL, NRG1, PDGFB, PHKG1, POU3F1, SCGB1A1, SEMA3B, SFTPB, SMAD6, SNCG, STC1, TCF3, TITF1, VEGF	45	Cellular Growth and Proliferation, Cellular Development, Tissue Development
	2	APOA1, APO3, AVP, C1QL1, CAMKB, CCKBR, CETP, CHGA, CRLF2, DGKQ, DNM2, EVPL, GAS, GFAP, GNAO1, GRIN1, GRIN2A, GRIN2D, IVL, KIAA1893, LDLR, OPRL1, POMC, PRKCE, PRKCG, PTPN5, RELN, SLC19A1, SRC, SYNGAP1, TCF7L2, TFAP2A, TFAP2C, TNRC6A, WWOX	45	Cardiovascular Disease, Organismal Injury and Abnormalities, Tissue Morphology
	3	CD19, CD28, CD209, CSF2, CSF2RA, CSF3R, CUL3, EGR1, EGR4, FYB, HGF, GNRPC, IL1RL1, IL5RA, IRF8, IRS4, ITGAX, LCP2, MAFF, NFE2, SLC6A6, SOCS3, SPI1, TG, WT1, ZNF224	45	Cellular Growth and Proliferation, Cellular Development, Hematological System Development and Function
HSV 5h	1	AMPD3, CCL2, CCL7, CCRN4L, CD14, CEACAM1, CXCL2, CXCL3, CXCL6, DSCR1, DUSP5, FUT7, GCH1, GDF15, GUCA2A, ICAM1, IL8, IL1B, IL1F6, IL1RN, IL6R, ITGAM, KLF10, LCN2, MUC5AC, NFKB1, NFKBIZ, PIM3, PRTN3, SDFR1, SELPLG, SLFN5, THBD, TNFAIP2, UGCG	45	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Cellular Movement
	2	ADRA1D, ARC, ATP8A2, BATF, BRD2, CCNT1, CEBPD, CYP17A1, CYP2E1, DUSP1, E2F2, EGR1, GADD45A, GADD45B, GADD45G, HLA-DQB1, HLA-DRA, IL6, IL19, IL1RL1, IRF1, JUNB, LIF, MHC2TA, NCOR2, NR1I2, NR4A1, OAS1, POMC, PPP1R15A, SOCS1, SOD2, SPEN, TFF2, XBP1	45	Cellular Growth and Proliferation, Gene Expression, Cell Death
	3	BCL2L11, CAPZB, CD19, CEBPE, CKS2, COX6A1, DPYSL5, DUSP4, DUSP6, EGR4, EIF4A1, EZH1, FOXO3A, HERC5, HUMMLC2B, ID1, IER3, KLF2, MATK, METAP2, MYC, MYCN, NGFB, NOL5A, NRG1, PHKG1, PTMA, RPL10, RPS12, SARDH, SGK, TNRC6A, UGT1A6, WT1, XIST	45	Cell Death, Reproductive System Disease, Cellular Development
	4	ATF3, BCL3, CAMK2A, CUL3, EDN1, EGFR, ELK4, ELL, EREG, FOS, FOSB, FOSL1, GAS5, HBEGF, HGF, IER2, IGHM, IGKC, IL11, IRX5, ITGB4, NTF3, PLAU, PLAUR, RPS6KA4, SLC19A1, SNX13, SYNGAP1, TACR1, TFAP2C, TLL2, TPSAB1, UBASH3A, VWF, WWOX	45	Cellular Growth and Proliferation, Cellular Movement, Cell-To-Cell Signaling and Interaction
	5	ATOH1, BMP7, CCL14, CDKN2A, CXCL1, CYR61, EVPL, F3, FOXA2, FOXC2, GTSE1, ID3, IVL, KIAA1036, LIPE, MAP3K8, NFKB2, NFKBIA, NR3C1, OPRL1, PDE8A, PNOC, PRKCE, TGS2, RELB, RPL10A, RUNX1, SEMA3B, SKP1A, SNCG, SNTB2, STC1, TBX3, TGIF, VEGF	45	Cellular Movement, Cancer, Cellular Development

Confirmation of micrarray data by real-time RT-PCR. A total of 40 samples with duplicates spanning a wide range of expression levels across different time points in the microarray experiments were selected for validation by real-time RT-PCR of fold-changes between virus-infected cells and mock-infected cells. Results are shown in Table 4. The genes with less than 2-fold relative gene expression change between virus-infected and mock-infected cells were all confirmed by real-time RT-PCR with the exception of IL1β, which was highly induced in HSV-1-infected cells at both 3 h and 5 h p.i. All genes with more than 2-fold change in microarray data were also confirmed to be up-regulated by real-time RT-PCR, but generally the extent of fold-change obtained from real-time RT-PCR data was significantly greater than data calculated from microarray hybridization signals.

Table 4. Validation of icroarray data with real-time RT-PCR. Data are represented as fold change (+/-SD) comparing virus infected samples to mock-infected samples. (-: Not changed or absent)

Gene family and gene name	GeneBank no.	Microarray fold change				Real-time PCR fold change			
		BV3h	BV5h	HSV3h	HSV5h	BV3h	BV5h	HSV3h	HSV5h
IRF-1	NM002198 202531_at	1.23	0.66	3.95	2.59	1.61 +/-0.08	0.82 +/-0.07	9.89 +/-0.54	4.14 +/-0.22
IL1β	NM_000576 205067_at	1.26	1.18	1.91	1.79	0.44 +/- 0.03	0.22 +/- 0.01	30.09 +/-1.65	17.84 +/-0.63
IL8 (CXCL8)	NM_000584 211506_s_at	-0.56	-1.11	31.46	6.90	0.94 +/-0.05	0.24 +/-0.01	30.59 +/-1.78	8.50 +/-0.44
(p105) (p50 precursor)	NM003998 209239_at 239876_at	1.35	1.11	3.51 1.53	1.77	1.01 +/-0.05	0.44 +/-0.04	1.94 +/-0.04	1.86 +/-0.17
NFKBIZ	NM_031419 223218_s_at	4.36	1.56	11.91	2.99	7.61 +/-0.37	3.05 +/-0.14	20.06 +/-2.72	13.50 +/-0.87
CXCL1	NM_001511 204470_at	-1.07	-1.32	20.82	8.30	0.77 +/-0.03	0.18 +/-0.01	25.67 +/-1.17	12.16 +/-0.62
CXCL2	M57731 209774_x_at	-	1.22	33.45	3.43	1.69 +/-0.06	0.69 +/-0.03	56.27 +/-2.01	10.89 +/-0.56
CXCL3	NM_002090 207850_at	1.08	1.06	23.50	6.93	1.31 +/-0.05	0.47 +/-0.02	46.65 +/-2.13	12.49 +/-0.88
CCL2	S69738 216598_s_at	-	1.09	5.30	6.73	0.57 +/-0.02	0.62 +/-0.03	5.83 +/-0.21	9.37 +/-0.48
IL6	NM_000600 205207_at	-1.64	-1.33	5.06	2.53	0.46 +/-0.03	1.19 +/-0.05	4.05 +/-0.24	9.25 +/-0.50

Part 2. Identification of virus-mediated human host cell responses at early time points of infection that target immune cells.

Host cell immune response genes regulated by B virus and HSV-1 at early times post infection. To identify immune response genes regulated during the initial wave of virus infection, genes with expression changes if at least 2-fold were sorted, and the hierarchical cluster analysis results are shown in Fig 6. Most of these genes that were involved in either innate or adaptive immune responses were up-regulated at 3 h or 5 h post HSV-1 infection. Only a few genes were found to be up-regulated in a border range of 2-fold change post B virus infection, i.e., EGR3 and NFKBIA gene. Furthermore, some immune responsive genes were found to be down-regulated in B virus infected cells, e.g., GADD45B, F3, IER3, SMAD7, IER3, SMAD7, FOS, HOXA9 and CDKN1B.

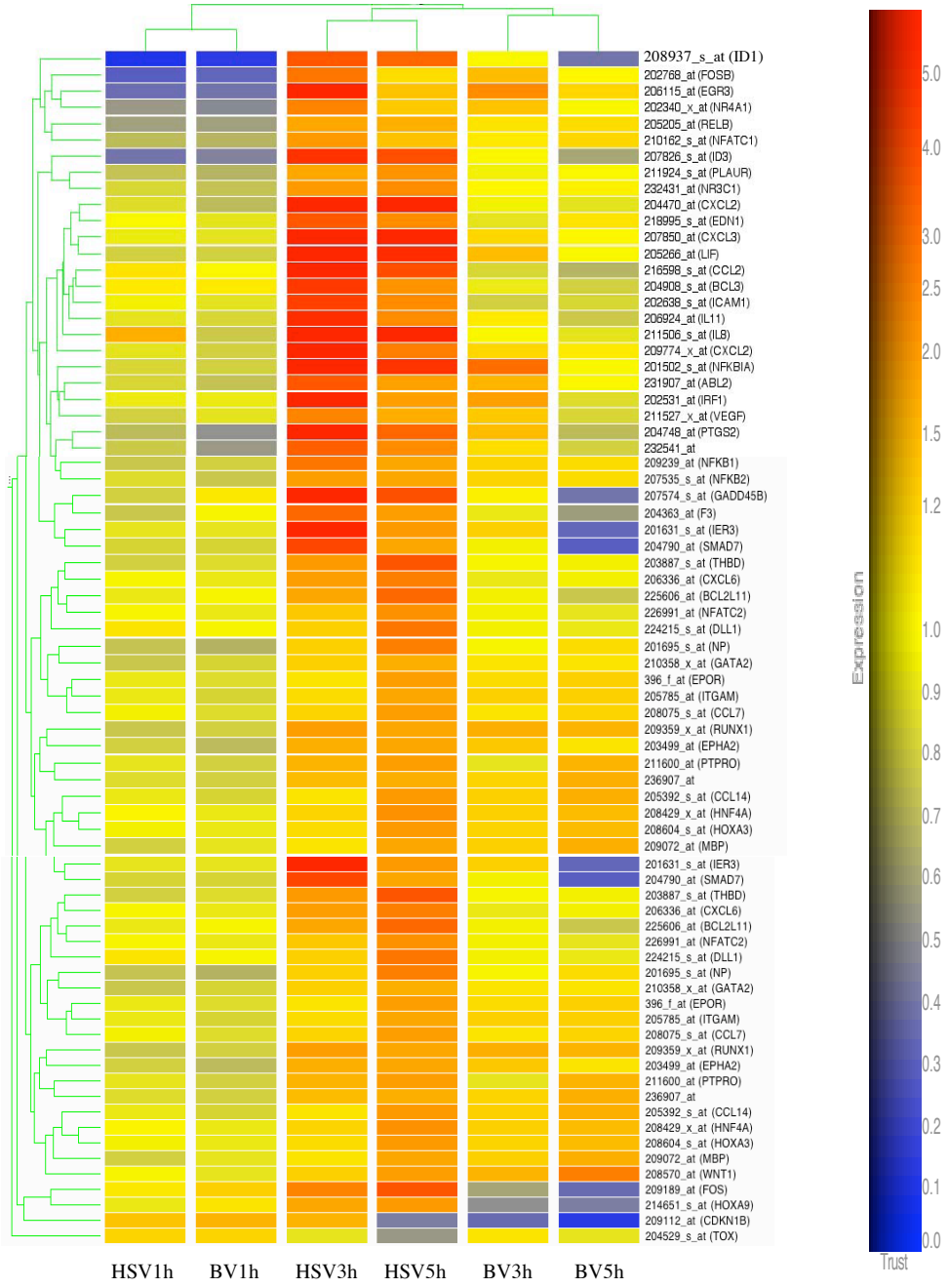

Fig 6. Hierarchical cluster analysis of immune responsive genes with at least 2-fold change genes. Each square in the heat map represents the average normalized expression level of a given gene per condition. Red represents increased expression and blue represents decreased expression compared to the mean of all normalized gene expression levels across all gene chips. Samples with similar gene expression profiles are clustered together. The color bar represents normalized expression scale.

IFN signaling pathway. IFNs are cytokines with antiviral and immunomodulatory functions. So far more than 300 genes have been identified as IFN-stimulated genes (41) (39) and provide part of the innate immune response. Each host cell gene that was involved in IFN signaling pathway and had significant changes in any of virus infected samples were therefore sorted and compared, the results of which are shown in Fig 7. Only less than 1% of IFN-related genes were found to have significant changes in HSV-1 infected cells and none of these genes were up-regulated in B virus infected cell. IRF-1 was initially described as a positive transcription factor for the regulation of type I IFNs (IFN-α/β) and IFN-inducible genes (105) (35). In our microarray data, IRF-1 was up-regulated by HSV-1, but not by B virus. Quantitative RT-PCR confirmed that IRF-1 had a 9.89-fold increase at 3 h post HSV-1 infection and had a 4.14-fold increase at 5 h p.i. relative to B virus (Table 4), each value greater than those observed in B virus infected HFF cells (1.61-fold at 3 h p.i. and 0.82-fold at 5 h p.i.). The IFNAR2 the gene encoding the type I IFN receptor, showed a 2.7-fold increase at 3 h post HSV-1 infection, but not in B virus infected cells. Interestingly, an IFN-signaling inhibitor called suppressor of cytokine signaling-3 (SOCS3) was also observed to have about 3-fold induction during the first 5 hours of HSV-1 infection period, but not in B virus infected cells.

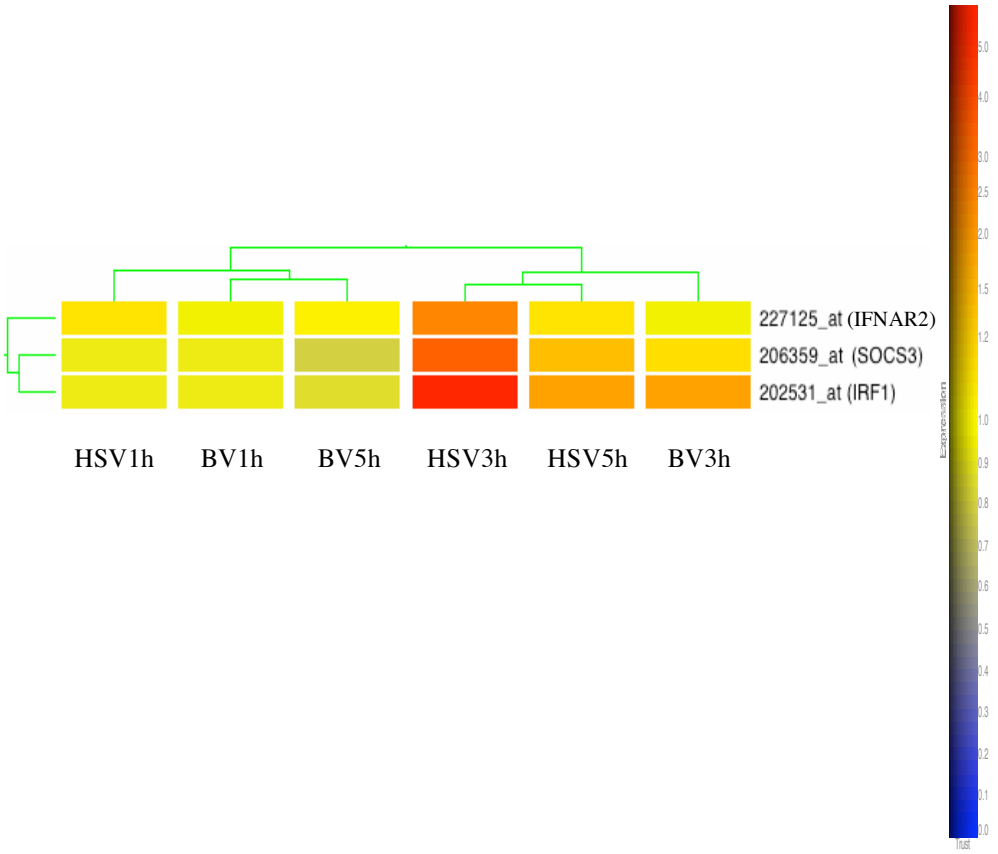

Fig 7. Hierarchical cluster analysis of IFN-related genes from microarray data in B virus and HSV-1 infected samples at 1, 3, and 5 h p.i. Each square represents the average normalized expression level of a given gene per condition. Red represents increased expression and blue represents decreased expression. Samples with similar gene expression profiles are clustered together. The color bar represents normalized expression scale.

Cytokines and chemokines. To identify which cytokines and/or chemokines were induced after infection with B virus or HSV-1, we initially measured expression of IL-6, IL-11, LIF, and chemokine genes CXCL1, CXCL2, CXCL3, CXCL6, CXCL8 (IL-8), CCL2, which were found to be up-regulated in HSV-1-infected cells compared to mock-infected cells, but not in B virus-infected cells (Fig 8). Chemokine genes, e.g., CXCL1, CXCL2, CXCL3 and CXCL8 (IL-8), were expressed, with ~20- to 33-fold change in HSV-1-infected samples at 3 h p.i., although the expression levels declined to ~ 3- to 8-fold at 5 h p.i. Strikingly, there were no cytokine or chemokine genes induced in B virus infected cells. Real time RT-PCR validated that HSV-1 not only induced CXCL1, CXCL2, CXCL3, CXCL8 (IL-8) and IL-6, but also increased the expression level of IL-1β. However, no cytokine or chemokine genes in B virus-infected samples showed significant differences, with less than 2-fold changes in expression levels compared to mock-infected samples (Table 4).

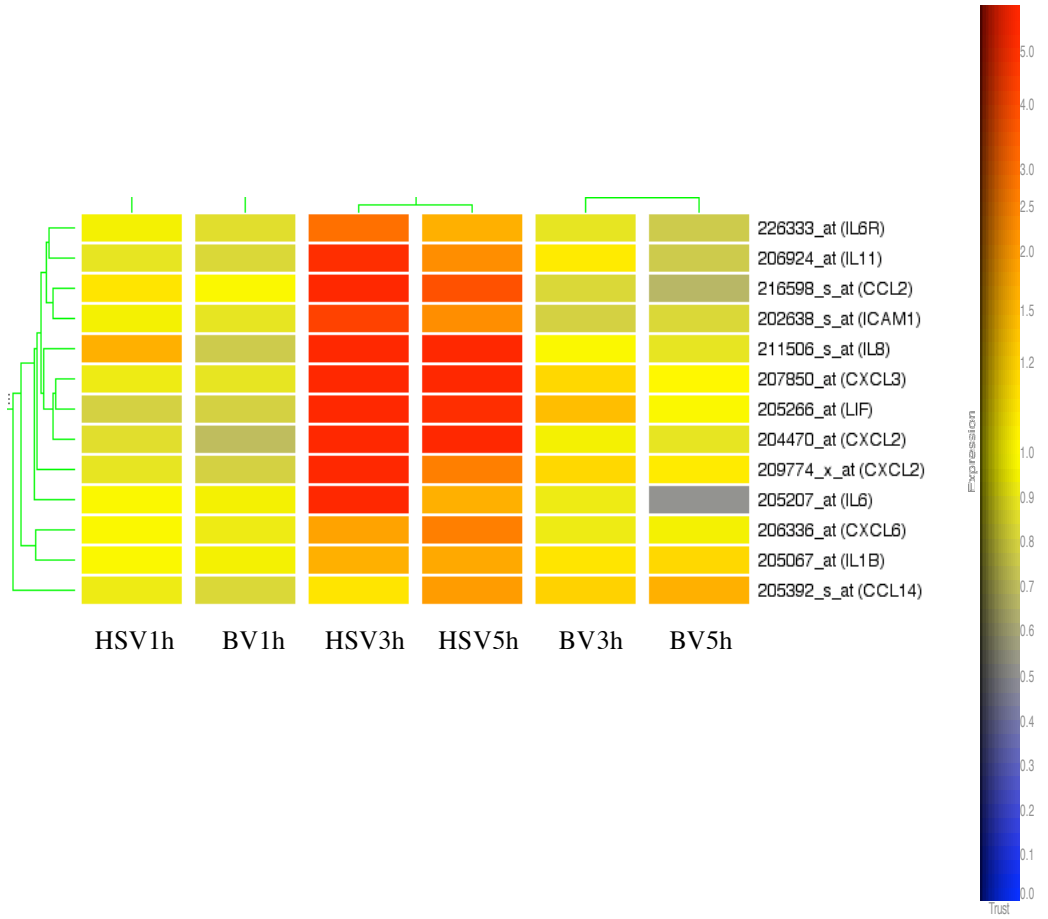

Fig 8: Hierarchical cluster analysis of cytokine genes from microarray data in B virus and HSV-1 infected samples at 1, 3, and 5 h p.i. Each square represents the average normalized expression level of a given gene per condition. Red represents increased expression and blue represents decreased expression. Samples with similar gene expression profiles are clustered together. The color bar represents normalized expression scale.

HSV-1 infected cell supernatants had greater neutrophil chemoattractant activity than B virus infected cell supernatants. To determine whether functional differences existed between virus-mediated cytokine or chemokine responses to target immune cells, neutrophil chemotaxis assays were used. In prior microarray datasets, a set of cytokines and chemokines genes, e.g. IL-1β, IL-6, IL-11 CXCL1, CXCL2, CXCL3, CXCL6, CXCL8 (IL-8), and CCL-2, were noted to have been induced in HSV-1-infected cells, but not in B virus-infected cells. Because most of these cytokines and chemokines produced from HSV-1-infected HFF cells had the ability to induce neutrophil chemotaxis activities, a neutrophil (PMN) transmigration assay was performed to assess the ability of cytokines and chemokines secreted from HSV-1-infected cells to facilitate neutrophil movement. In contrast, B virus-infected cells were not expected to have detectable neutrophil chemotaxis activites. To our surprise, B virus infection was associated with less neutrophil chemoattractant activity compared to mocked-infected cell supernatants (Fig 9), suggesting that B virus inhibited neutrophil transmigration. Comparing neutrophil chemoattractant activity between B virus and HSV-1 infected cell supernatants, however, revealed that HSV-1 induced signigicantly greater chemoattractant activity, as predicted by the microarry data neutrophil transmigration rate was further quantified by measuring the activity of myeloperoxidase (MPO), a unique enzyme present in neutrophils (Fig 10). Although we observed less neutrophils transmigration in B virus-infected cell supernatants compared to mocked-infected cell supernatants (Fig 9), there was no significant difference in neutrophil transmigration rate between B virus- and mock-

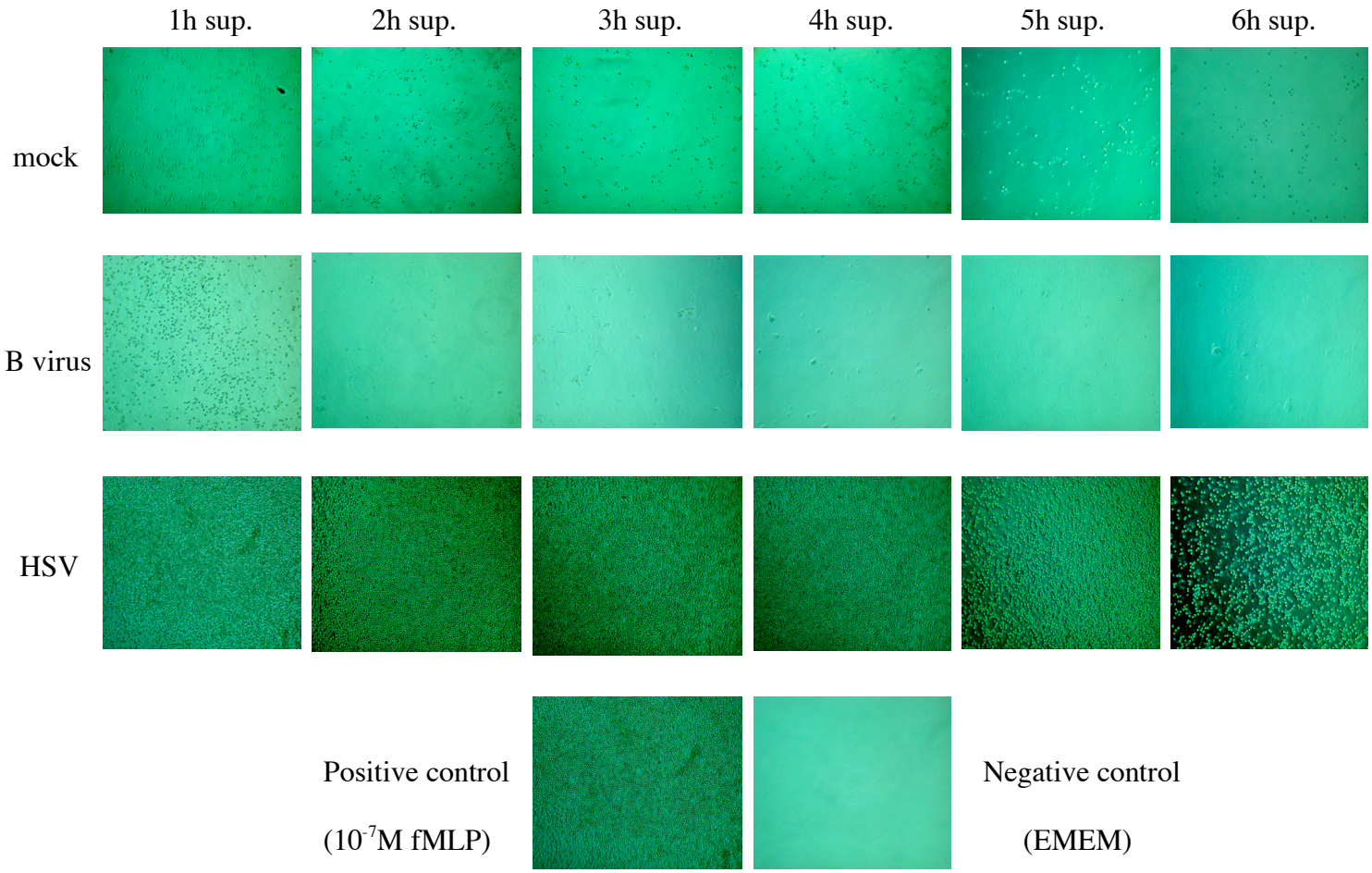

Fig 9. Neutrophil transmigration towards supernatants from B virus, HSV-1 and mock infected HFFs. Supernatants were harvested from cultures of infected or uninfected HFFs hourly. In the transwell setups, freshly prepared human neutrophils were added to 5 μm pore size filters and virus infected cell supernatants were added in the bottom wells. The migration of neutrophils across the transwells was photographed from the receiver wells. All supernatants samples were tested in duplicate wells. (EMEM: Eagles minimum essential medium; Sup.: supernatant)

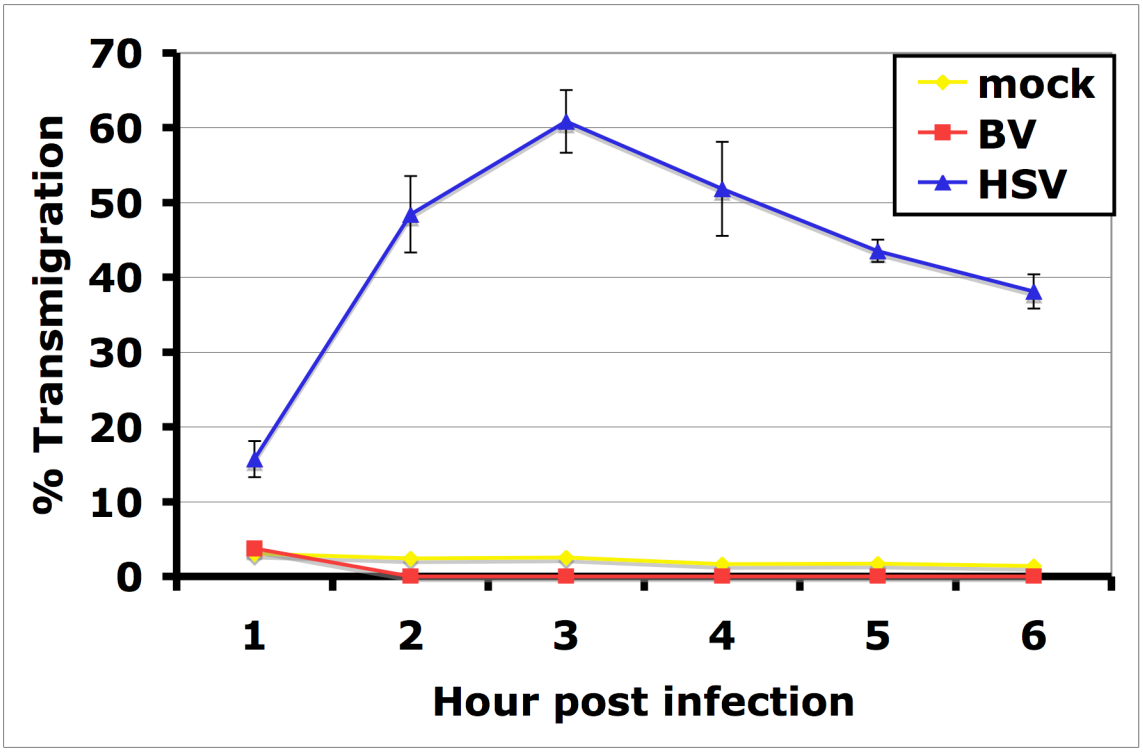

Fig 10. Quantification of transmigrated neutrophil numbers in B virus, HSV-1 and mock infected cell supernatants. Supernatants were harvested from cultures of infected or uninfected HFFs hourly and tested in a transmigration assay. The % of neutrophils transmigrating across the transwells was quantified by measuring MPO activity. All supernatant samples were tested in duplicate wells.

infected cell supernatants according to unpaired student t-test statistical analysis ($P>0.05$).

HSV-1, but not B virus induced IL-8 production. To further identify what caused virus infected cell supernatants to have neutrophil chemoattractant activity, we measured IL-8 by ELISA because this chemokine was one of the major mediators of the inflammatory response and functions as a chemoattractant. According to our previous microarray data, elevated IL-8 gene expression levels had been found in HSV-1-infected HFF cells (31-fold increased at 3 h p.i. and 7-fold increased at 5 h p.i.), but not in B virus-infected HFFs (0.6-fold decreased at 3 h p.i. and 1.1-fold decrease at 5 h p.i.). The IL-8 ELISA results (Fig 11) showed that B virus- and mock-infected cells produced similar IL-8 amount during the first 6 hour infection period. However, HSV-1 induced higher IL-8 than mock- and B virus-infected cells during all 6 hours post infection. This data supported our microarray data and may explain why more chemoattractant activity was found in HSV-1 infected cell supernatants than B virus infected cell supernatants in the above neutrophil transmigration experiment.

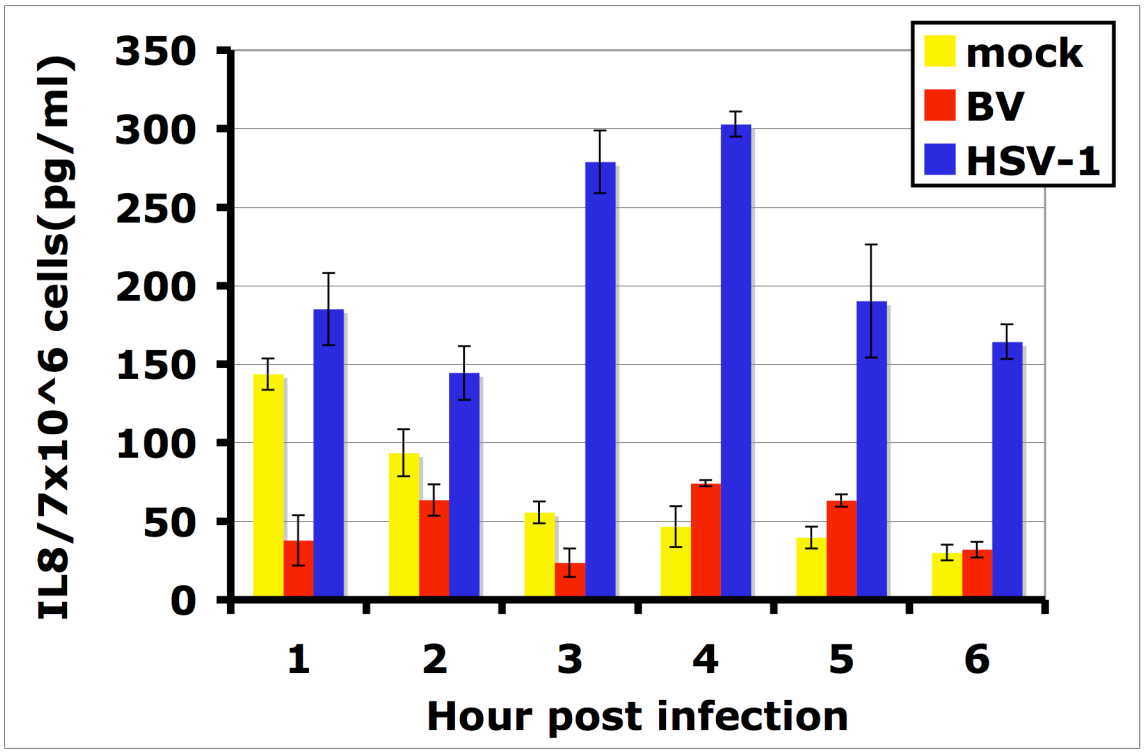

Fig 11. Comparison of IL-8 productions in B virus, HSV-1 and mock-infected cell supernatants. Supernatants were harvested from cultures of infected or uninfected HFFs hourly. IL-8 concentrations were measured by ELISA. (n=3). Values are the means ± standard deviations.

The inhibitory effects of virus infected cell supernatants to neutrophil transmigration. Since there was no neutrophil transmigration activities observed in B virus-infected cell supernatants except in 1 h p.i. cell supernatants, we were interested to know if it could be due to the presence of any inhibitor besides the fact that no chemoattractants were found in B virus-infected cell supernatants (Fig 9 & Fig 10). Neutrophil transmigration inducers IL-8 and fMLP were therefore mixed with virus infected cell supernatants (n=4) and the neutrophil transmigration rates toward IL-8 or fMLP treated samples were compared to the controls that only had IL-8 or fMLP, but no virus infected cell supernatants (Fig 12, 13 and 14). The result showed that B virus-infected cell supernatants caused both IL-8 and fMLP-mediated neutrophil transmigration rate to be reduced, and this inhibitory effect was greater at later time points (Fig 15 & 16). HSV-1-infected cell supernatants showed the same trend as B virus in inhibiting IL-8-mediated neutrophil transmigration (Fig 15), but HSV-1 had a greater inhibitory effect in fMLP-mediated neutrophil transmigration at the earliest time point (1 h p.i.) and less inhibitory effect at a later time point (5 h p.i.) (Fig 16). Mock-infected cell supernatants also showed reduced IL-8 and fMLP-mediated neutrophil transmigration rates, which suggested that there was a host cell-derived inhibitor present in the cell culture supernatants.

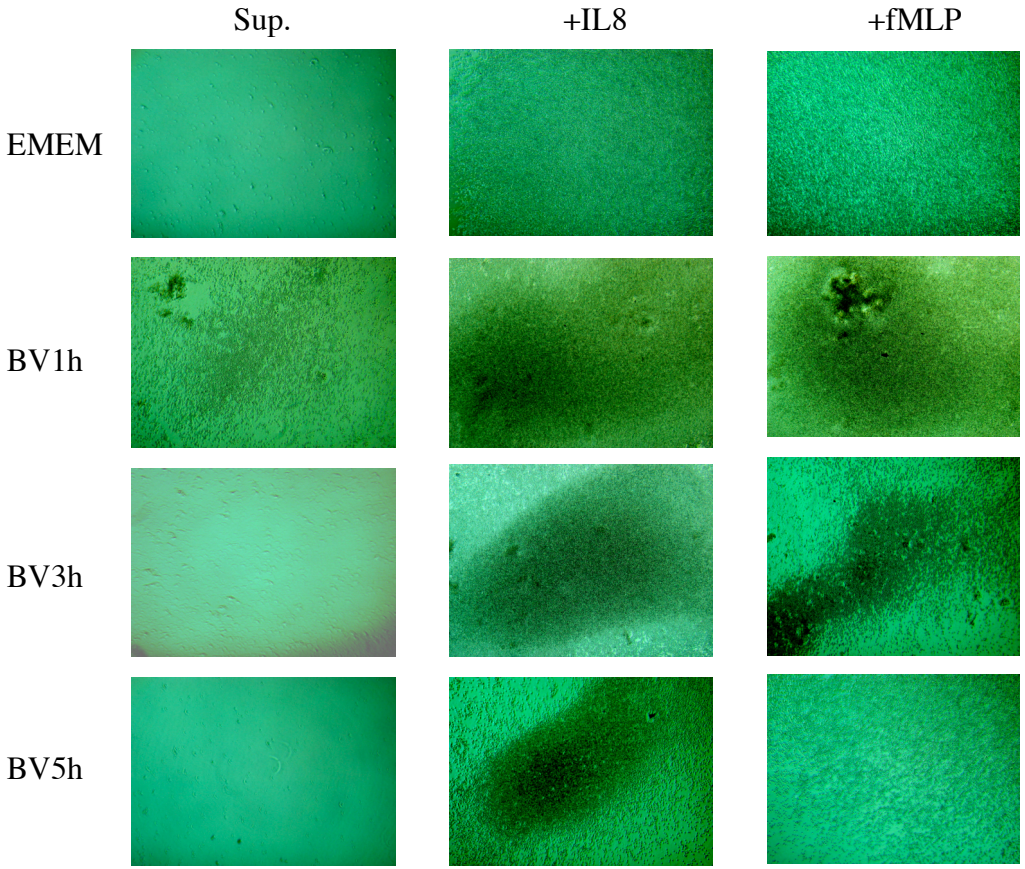

Fig 12. B virus infected cell supernatants-mediated inhibition to IL-8- and fMLP-induced neutrophil transmigration. The neutrophil transmigration inducers, 0.2 μM f-MLP or 200 ng/ml IL-8, were added into the lower chamber of the transwells setups, in addition to the presence of tested B virus-infected cell supernatants. Supernatants were harvested from cultures of B virus infected HFFs at 1 h, 3 h and 5 h p.i. The migration of neutrophils across the transwells was photographed from the receiver wells. All supernatant samples were tested in duplicate wells.

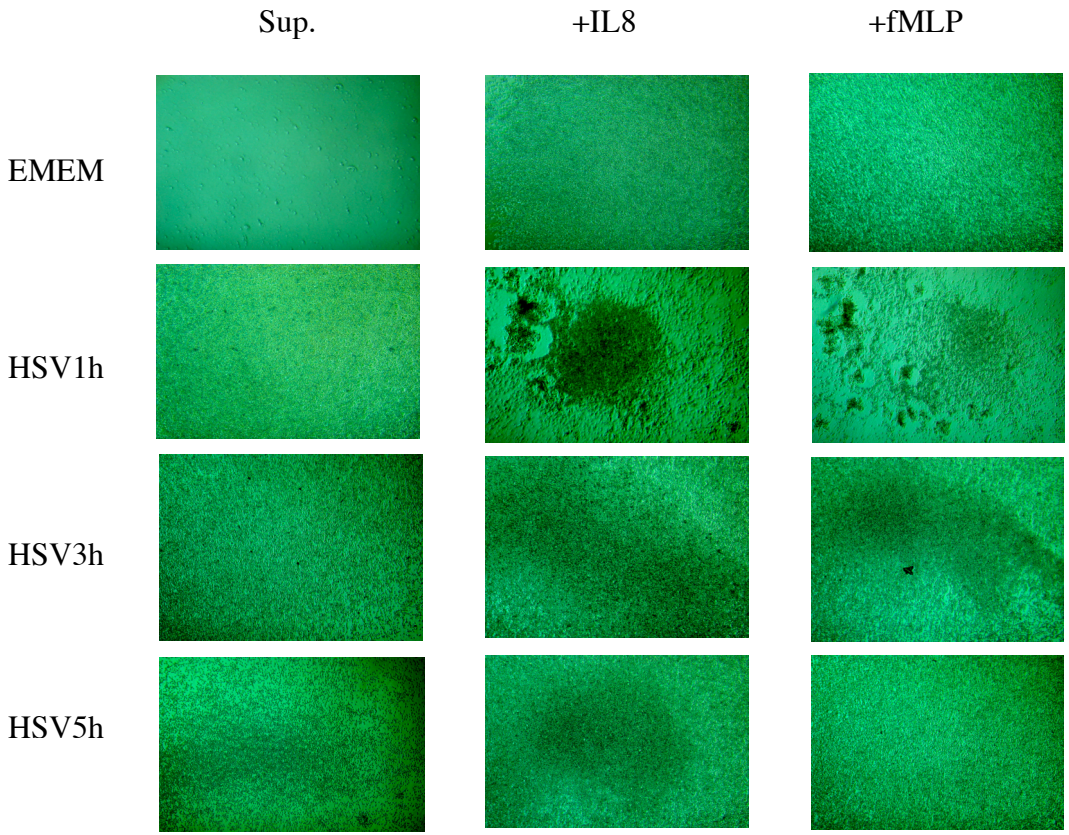

Fig 13. HSV-1 infected cell supernatants-mediated inhibition to IL-8- and fMLP-induced neutrophil transmigration. The neutrophil transmigration inducers, 0.2 μM f-MLP or 200 ng/ml IL-8, were added into the lower chamber of the transwells setups, in addition to the presence of tested HSV-1-infected cell supernatants. Supernatants were harvested from cultures of HSV-1 infected HFFs at 1 h, 3 h and 5 h p.i. The migration of neutrophils across the transwells was photographed from the receiver wells. All supernatant samples were tested in duplicate wells.

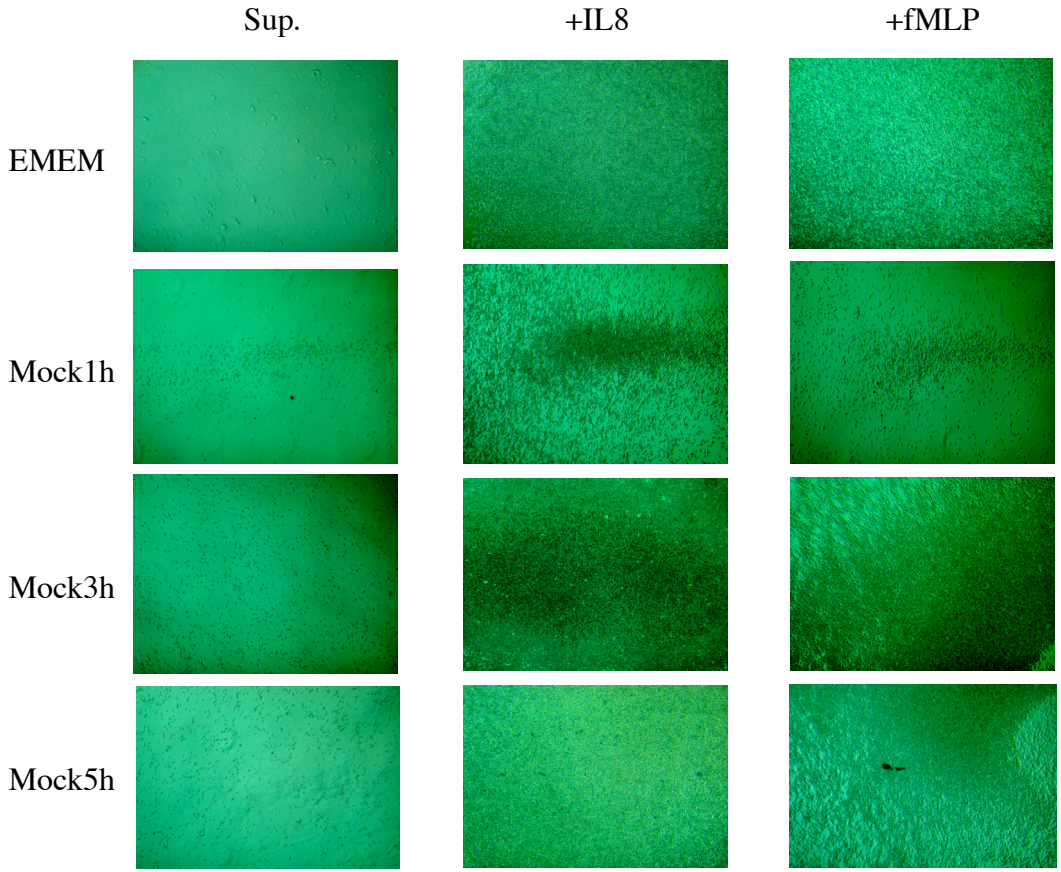

Fig 14. Mock infected cell supernatants-mediated inhibition to IL-8- and fMLP-induced neutrophil transmigration. The neutrophil transmigration inducers, 0.2 μM f-MLP or 200 ng/ml IL-8, were added into the lower chamber of the transwells setups, in addition to the presence of tested mock-infected cell supernatants. Supernatants were harvested from cultures of uninfected HFFs at 1 h, 3 h and 5 h p.i. The migration of neutrophils across the transwells was photographed from the receiver wells. All supernatant samples were tested in duplicate wells.

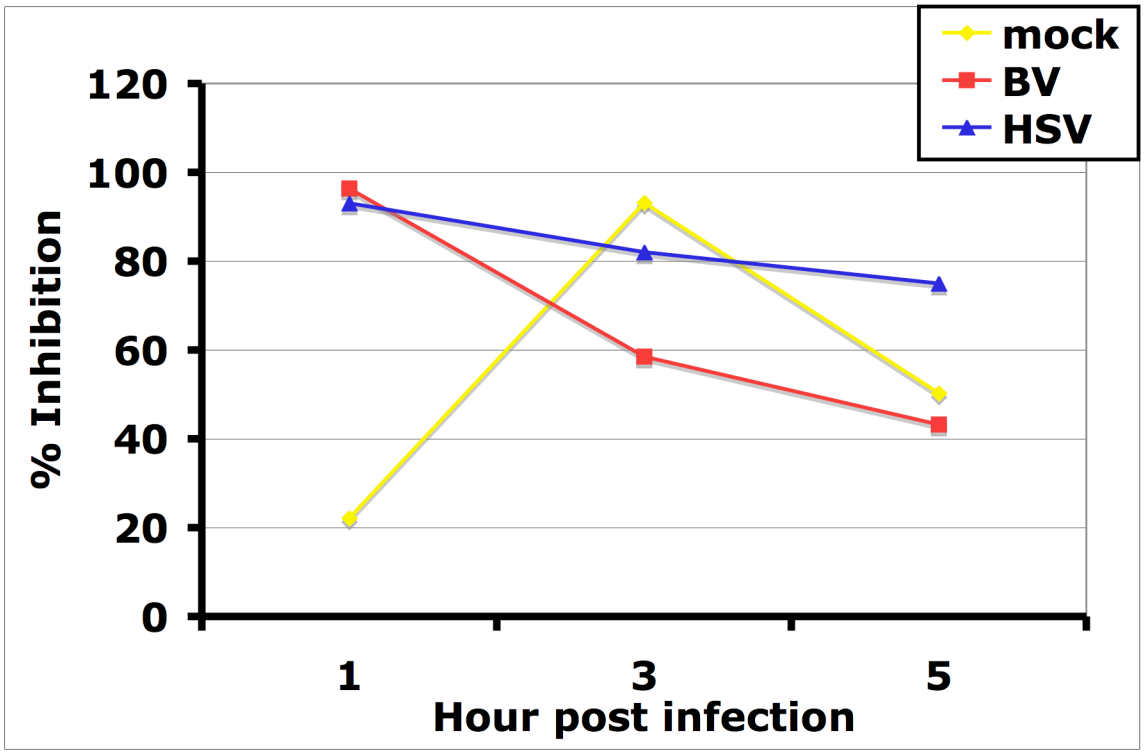

Fig 15. Quantitiation of virus infected cell supernatants mediated inhibition to IL-8-induced neutrophil transmigration. The neutrophil transmigration inducers, 200 ng/ml IL-8, were added into the lower chamber of the transwells setups contaning mock-, or B virus- or HSV-1-infected cell supernatants. Supernatants were harvested from cultures of B virus, HSV-1 infected or mock-infected HFFs at 1 h, 3 h and 5 h p.i. The numbers of migrated neutrophils across the transwells were quantified by measuring MPO activity (n=4).

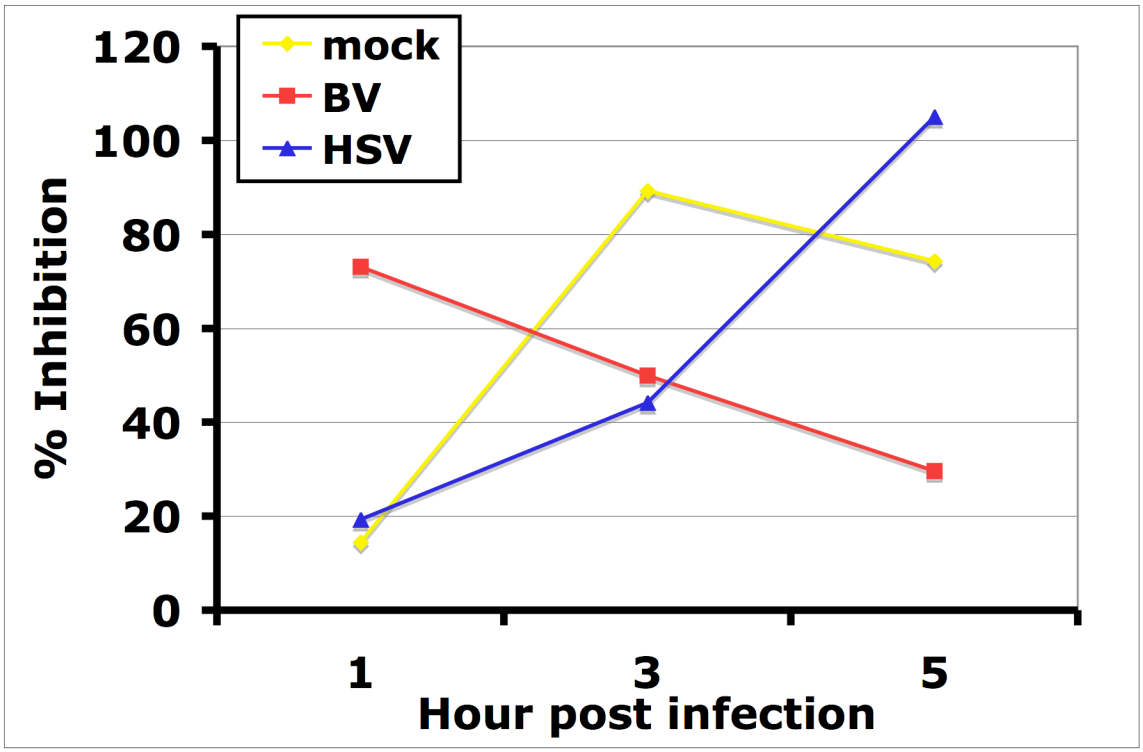

Fig 16. Quantitation of virus infected cell supernatants mediated inhibition to fMLP-induced neutrophil transmigration. The neutrophil transmigration inducers, 0.2 μM f-MLP, were added into the lower chamber of the transwells setups containing mock-, or B virus- or HSV-1-infected cell supernatants. Supernatants were harvested from cultures of B virus, HSV-1 infected or uninfected HFFs at 1 h, 3 h and 5 h p.i. The numbers of migrated neutrophils across the transwells was quantified by measuring MPO activity (n=4).

Cytokine gene expression in infected human cells was involved in NF-κB pathway. The clear differences in the gene regulation responses of HFF cells to infection with HSV-1 or B virus suggested that virus infection had resulted in virus type-specific host cell interactions (Fig 6 & 8). Ingenuity™ pathway analysis tool was used to construct a biological network in order to connect the molecular relationships among transcripts involved in innate immunity to reveal disparate outcomes of each virus infection. Figure 17 illustrates the interconnectedness of HSV-1 up-regulated host genes at 3 h p.i.; induction of NF-κB transactivated cytokines is apparent. A similar, but functionally different network, was also found in B virus-infected cells, the expression levels of these genes which composed this network showed less than a 2-fold change, except for NFKBIZ.

Nuclear translocation of NF-κB p50. To test the hypothesis that the failure of cytokine gene induction in B virus-infected cells was due to the inhibition of NF-κB activation, the nuclear translocation of NF-κB p50 was quantified to measure the activation status in the nucleus after B virus and HSV-1 infection. The activation of NF-κB p50 was analyzed by Cellomics ArrayScan cytometer, which measured levels of NF-κB p50 translocation from the cytoplasm to nucleus, and represented these measurements as the difference in average fluorescent staining intensity between the nucleus and cytoplasm.

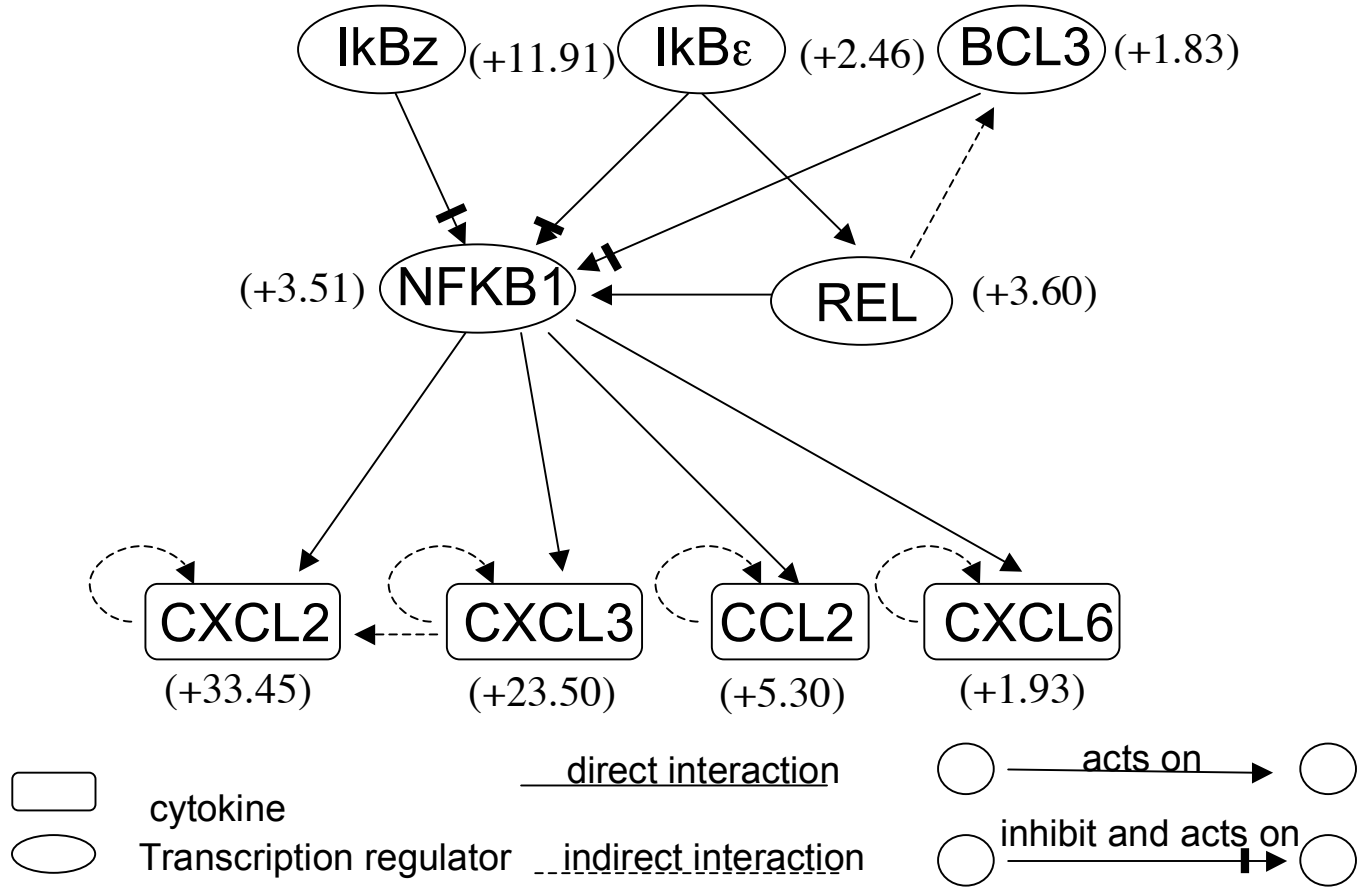

Fig 17. Biological network for NF-κB regulated immune responsive genes generated from HSV-1 3 h p.i. samples. The number next to each gene represent the fold change ratio comparing virus infected to mock-infected samples.

NF-κB can be activated by TNF-α phosphorylation dependent-ubiquitination and degradation of inhibitor of IκB proteins. Therefore, TNF-α-treated HFFs were used as a positive control for NF-κB p50 nuclear translocation, and the results are shown in Figure 18. NF-κB p50 was also activated by both B virus and HSV-1 (Fig 19). The amount of NF-κB p50 translocation from cytoplasm to the nucleus in B virus and HSV-1 infected cells was similar at 1 h, 2 h, 5 h and 6 h p.i. However, HSV-1 induced more NF-κB p50 nuclear translocation than B virus at 3 h and 4 h p.i.

DNA binding activity of NF-κB p50. To examine if the DNA binding activity of NF-κB p50 was inhibited and therefore caused cytokine gene expression blocked in B virus infected cells, nuclear extracts prepared from B virus, HSV-1 and mock-infected cells were incubated with a probe containing an NF-κB p50 consensus binding site bound to ELISA plates. To rule out possible cross-contamination of nuclear extracts by cytoplasmic contents, cytoplasmic extracts from infected cells were also tested for NF-κB p50 DNA binding activity, and no NF-κB p50 DNA binding activity was observed (data not shown). According to current understanding, NF-κB p50 present in the cytoplasmic fraction is bound by its inhibitor protein IκB, and will not be available to bind DNA too.

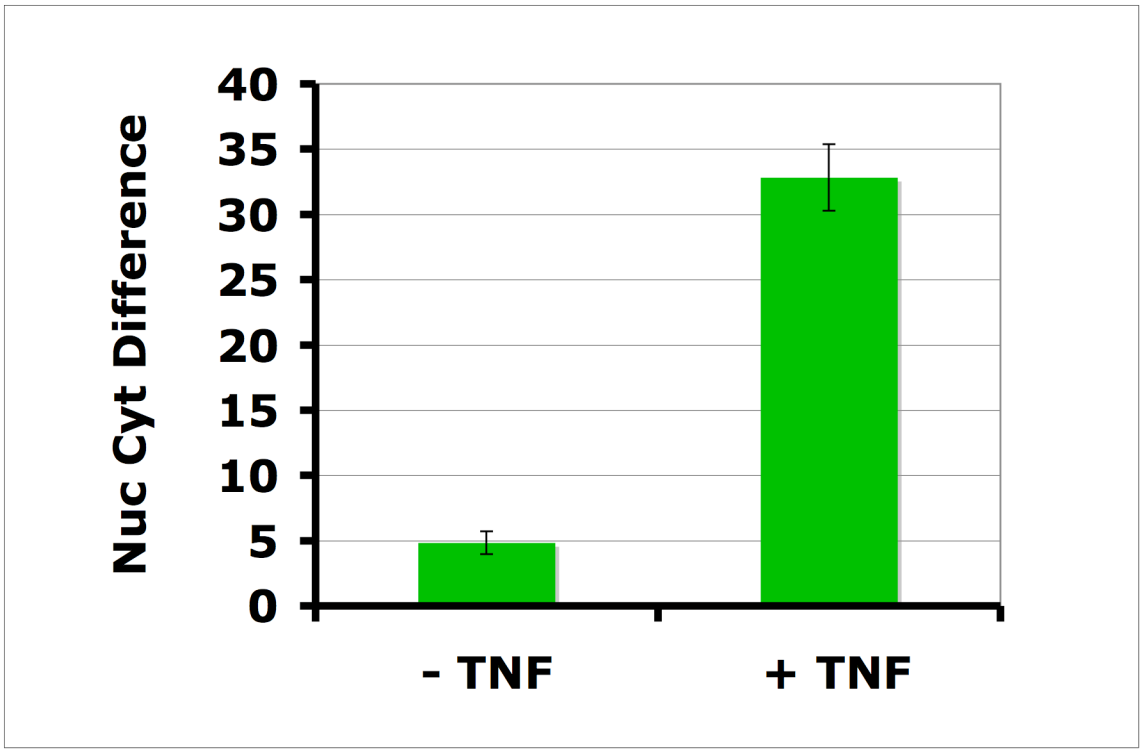

Fig 18. Nuclear translocation NF-κB p50 in TNF-α-treated HFFs. HFFs cells were treated with TNF-α (0.1μg/ml) for 1 h and the amounts of NF-κB p50 translocation from the cytoplasm to nucleus is measured by Cellomics ArrayScan cytometer and represented as the difference in average fluorescent staining intensity between the nucleus and cytoplasm. Data are the means ± standard deviations (n = 500).

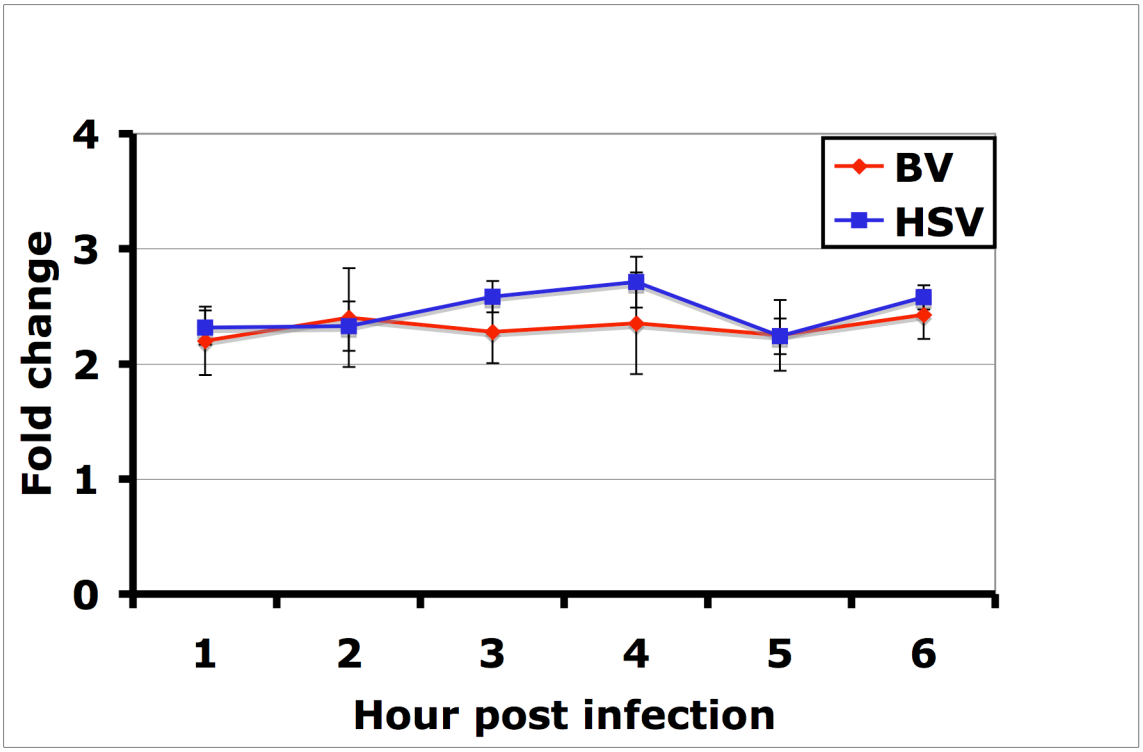

Fig 19. Nuclear translocation NF-κB p50 in B virus and HSV-1 infected HFFs. HFFs were B virus- or HSV-1- or mocked-infected at m.o.i. of 10 and the amounts of NF-κB p50 translocation from the cytoplasm to nucleus is measured by Cellomics ArrayScan cytometer and represented as the difference in average fluorescent staining intensity between the nucleus and cytoplasm. The ratio of nuclear to cytoplasmic fluorescence compared to that of mock-infected cells is calculated and the data are the means ± standard deviations (n = 500).

HSV-1 infection induced high levels of NF-κB p50 DNA binding activity, approaching the levels generated with the TNFα control (Fig 20). In B virus-infected cells, however, levels of NF-κB promoter binding activity mostly remained at mock levels. This suggests that one possible mechanism of the cytokine gene blockage in B virus-infected cells is due to the inhibition of NF-κB p50 DNA binding activity. These data collectively support the hypothesis under investigation based on the differences between HFF cells infected with B virus and the closely related family member HSV-1 with regard to regulation of NF-κB dependent innate responsive genes expression.

Tanscription factor genes. All of the transcription factors other than those in the NF-κB pathway and IFN signaling pathway regulated by B virus and HSV-1 are shown in Figure 21. HFF cells infected with B virus were observed to show no induction of any transcription factors within the first 5 hours; however, HSV-1 was observed to up-regulate 45 transcription factors with at least 2-fold change during the first five hours p.i.

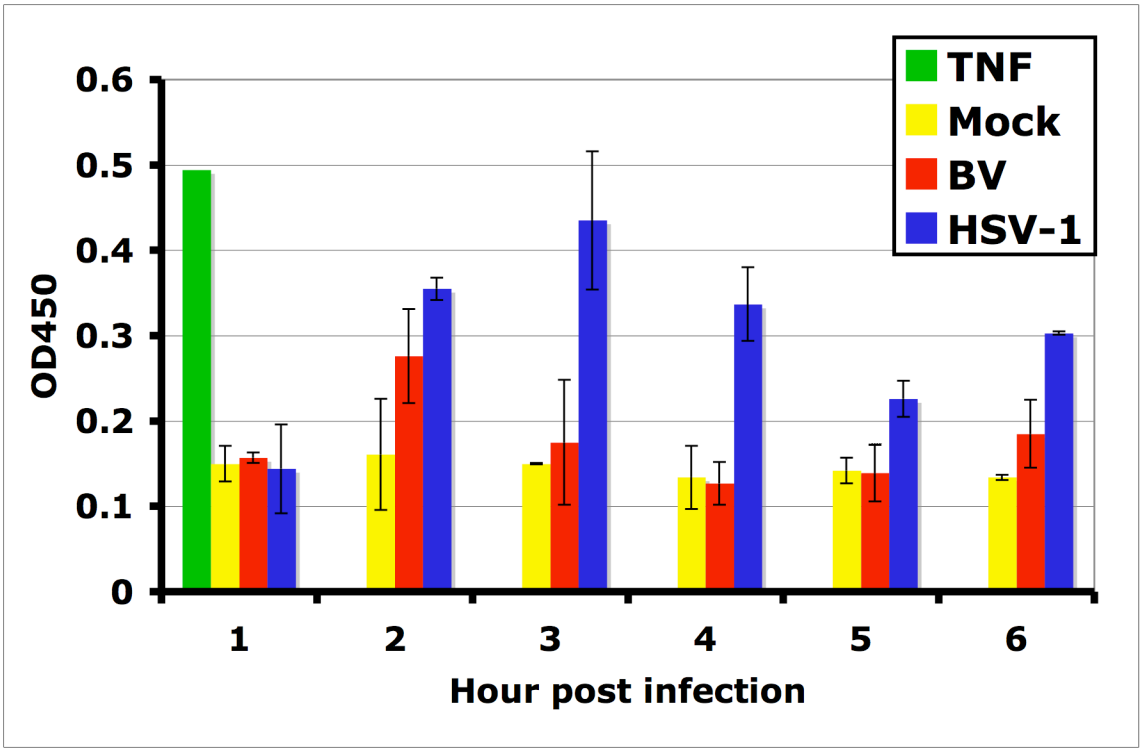

Fig 20. NF-κB p50 DNA binding activity in B virus and HSV-1 infected HFF cell nuclear preparations. NF-κB p50 DNA binding activity was measured by ELISA. Data are the means ± standard deviations (n = 3).

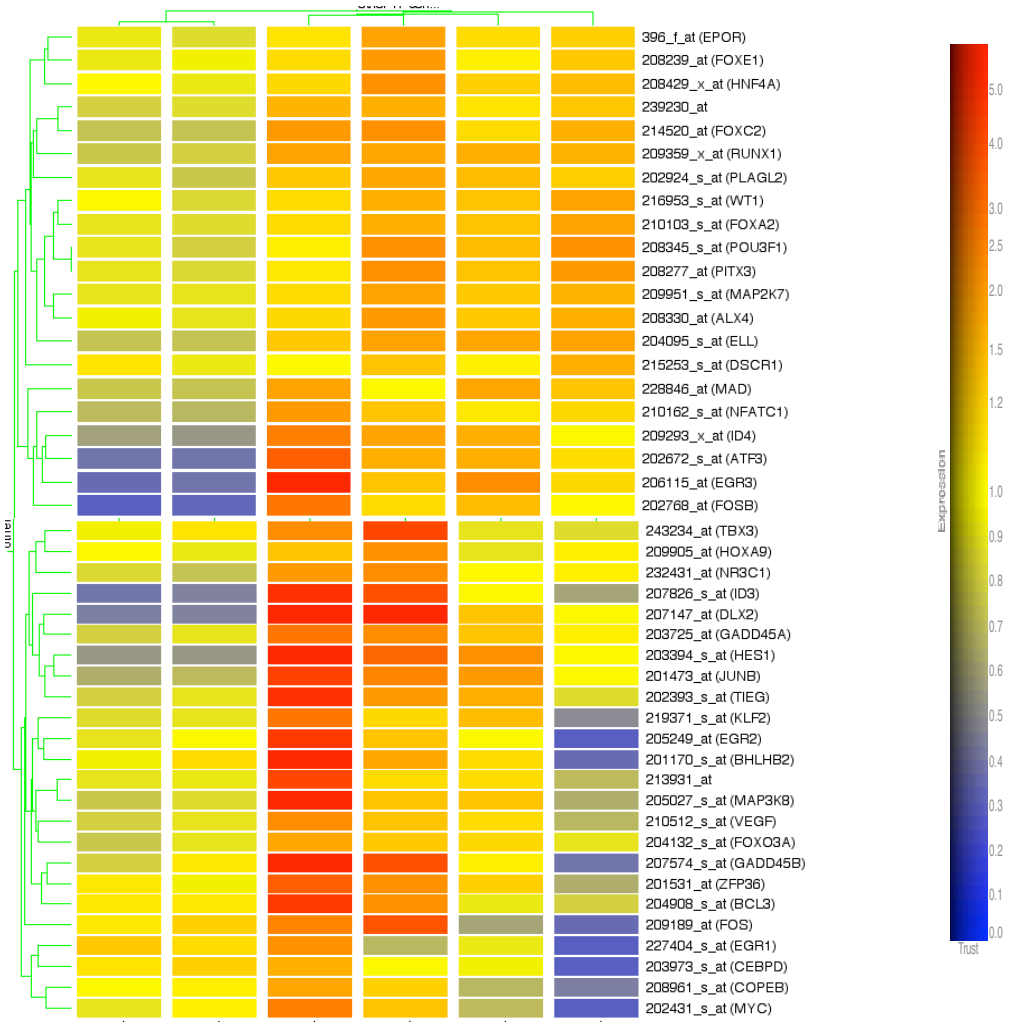

Fig 21: Hierarchical cluster analysis of transcription factor genes from microarray data in B virus and HSV-1 infected samples at 1, 3, and 5 h p.i. Each square represents the average normalized expression level of a given gene per condition. Red represents increased expression and blue represents decreased expression. Samples with similar gene expression profiles are clustered together. The color bar represents normalized expression scale.

Part 3. Design of blocking strategies to circumvent early events in the infected cell to modulate immune responses.

B virus inhibits NF-κB pathway by inducing a nuclear protein IkBζ. To identify if there was any gene specifically induced in B virus infected cells that might cause the inhibition of NF-κB p50 DNA binding activity, hierarchical cluster analysis was used subsequently to sort all transcripts found to have significant changes related to the NF-κB pathway (Fig 22). Examination of NF-κB gene p100 (p52 precursor), p105 (p50 precursor), REL (c-REL) and RelB revealed fold-increases of 2.21, 3.51, 3.61, 2.39, respectively, in HSV-1-infected HFF at 3 h p.i. None of these NF-κB genes was induced by B virus infection at 1, 3 or 5 h p.i. While the NF-κB negative regulators, viz., IκBζ (NFKBIZ), IkBε (NFKBIE), IκBα (NFKBIA), and BCL-3 were induced in HSV-1-infected cells, IκBζ (NFKBIZ) was found as the only NF-κB negative regulator that was highly induced in B virus- infected cells. Real-time PCR confirmed that B virus inducible IκBζ (NFKBIZ) had a 7.61-fold increase at 3 h p.i., and 3.05-fold increase at 5 h p.i. (Table 4). Comparatively, HSV-1 inducible IκBζ (NFKBIZ) had a 20.26-fold increase at 3 h p.i., and 13.50-fold increase at 5 h p.i.

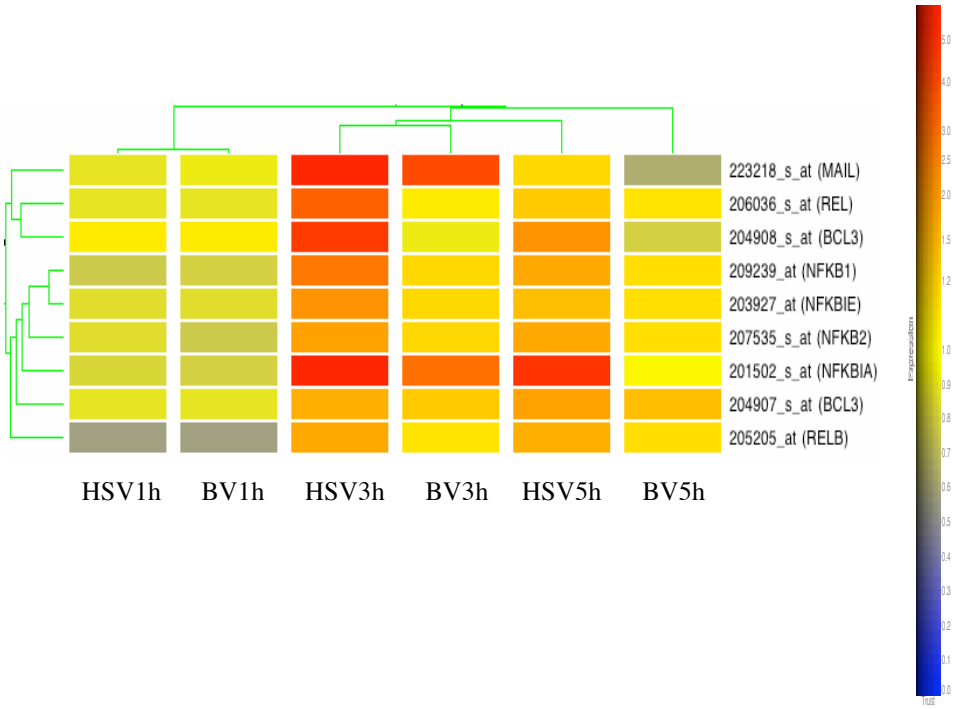

Fig 22: Hierarchical cluster analysis of NF-κB pathway related genes from microarray data in B virus and HSV-1 infected samples at 1, 3, and 5 h p.i. Each square represents the average normalized expression level of a given gene per condition. Red represents increased expression and blue represents decreased expression. Samples with similar gene expression profiles are clustered together. The color bar represents normalized expression scale. (MAIL gene = NFKBIZ gene)

B virus and HSV-1 induce IκBζ mRNA in infected HFF cells. To monitor the IκBζ mRNA expression levels in B virus infected cells during the first six hours of infection, relative changes of IκBζ mRNA levels between virus- and mock-infected human foreskin fibroblast (HFF) cells were quantified using real-time PCR (Fig 23). IκBζ mRNA expression levels in HSV-1 infected cells were also measured as a control. In B virus infected cells, IκBζ mRNA expression level increased by 3 h p.i. peaking at 4 h p.i., after which expression levels declined. Similar expression profiles were observed in HSV-1 infected cells, but the induction kinetics of the HSV-1 IκBζ mRNA was ~ 1 h faster than that observed in B virus infected cells. The IκBζ mRNA level in HSV-1-infected cells began to increase at 2 h p.i., reaching maximal levels by 3 h p.i., and then declined shortly thereafter. Both viruses induced increases in expression levels of IκBζ, albeit at different time points following infection.

B virus and HSV-1 induce IκBζ protein accumulation in the nuclei. Since mRNA expression is not always translated into protein, IκBζ protein translation was investigated by immunofluorescent staining, and images were acquired by confocal microscopy (Fig 24 & 25). In both B virus and HSV-1-infected HFF cells, IκBζ could be observed to be localized inside the nucleus within 2-6 hours following infection. Interestingly, IκBζ was not evenly distributed in the nucleus. It aggregated in distinct punctate-like structures and tended to be observed at the nuclear border. This result was similar to published description (155) of the subcelluar localization of IκBζ and reports that IκBζ co-localized with the nuclear corepressor SMRT (silencing mediator of retinoid and thyroid hormone

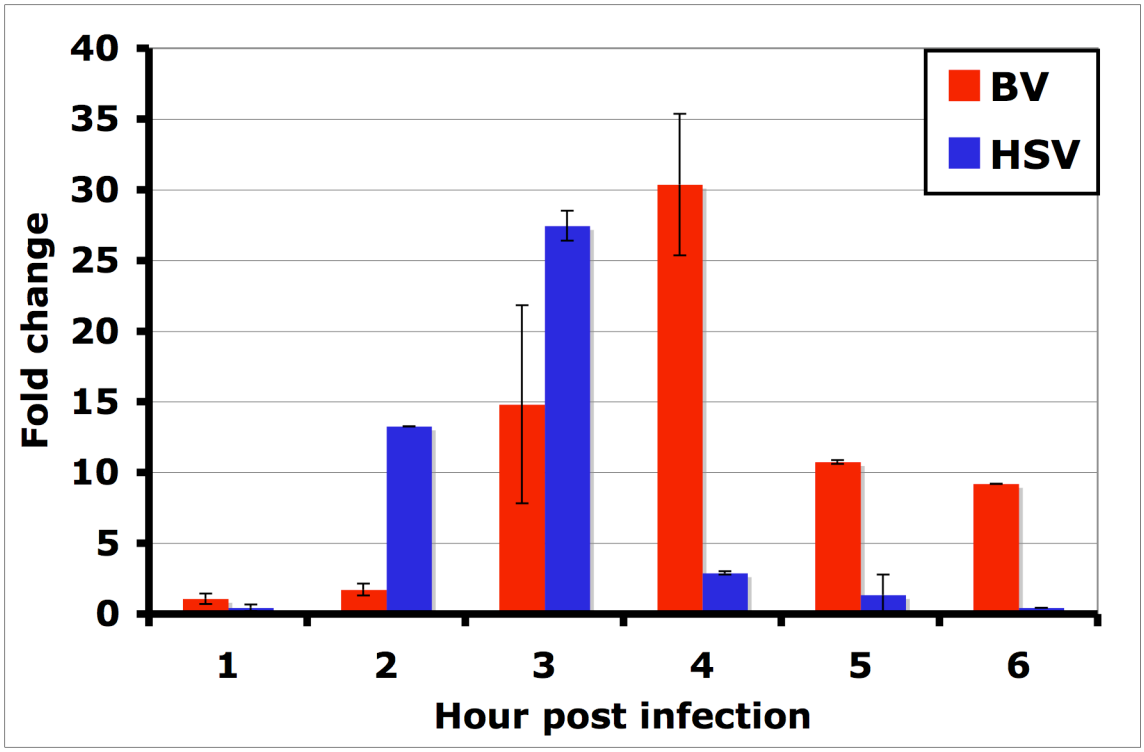

Fig 23. Kinetics of IkBζ mRNA level corresponding to B virus and HSV-1 infection. Total RNA extracted from B virurs-, HSV-1- and mocked infected cells at 1, 2, 3, 4, 5 ,6 h p.i. was used as a templte to generate cDNA. Levels of IkBζ mRNA were quantfied by real-time PCR and relative changes in gene expression were determined by using the relative standard curve method.

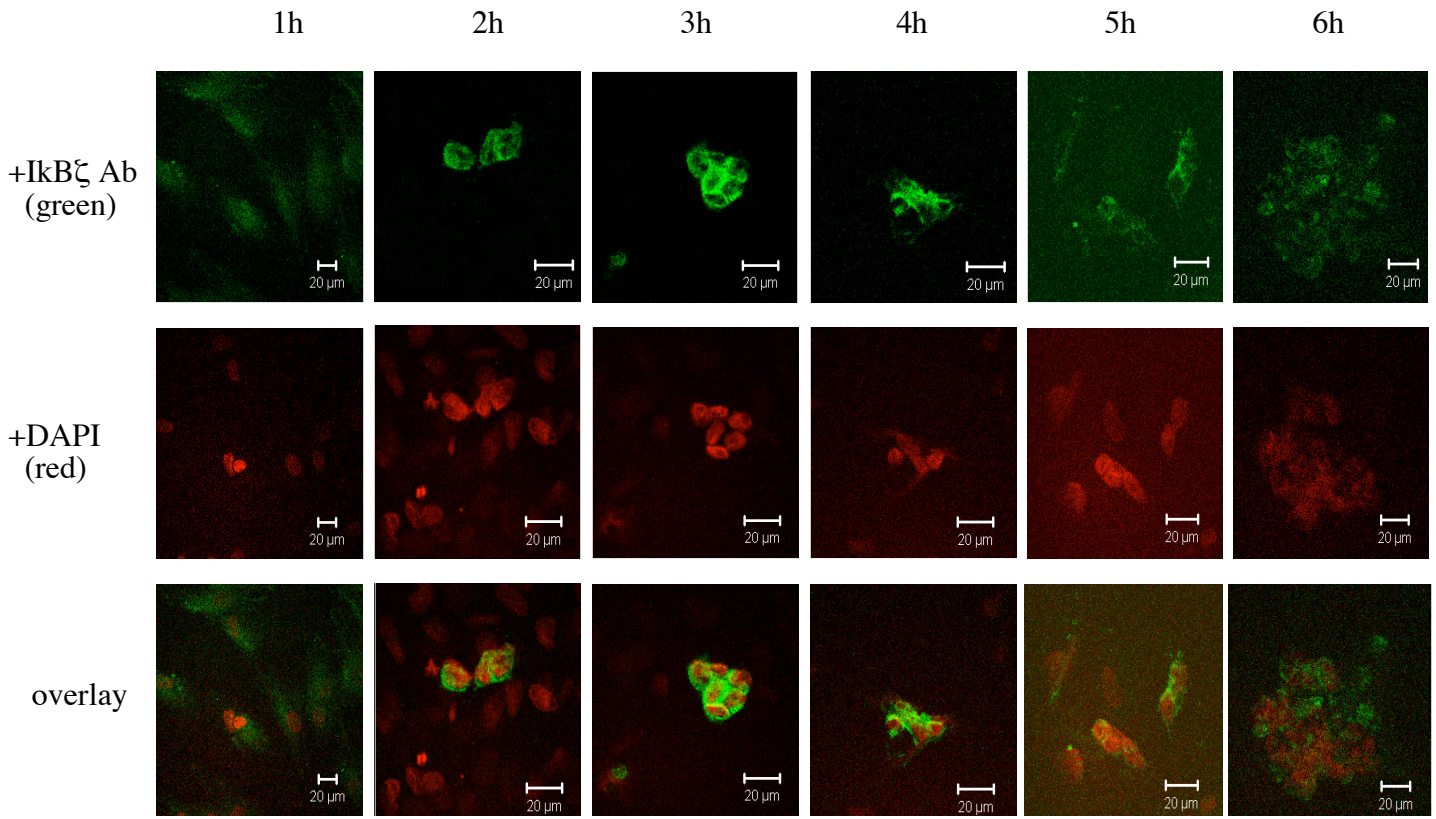

Fig 24. Immunofluorescent staining of IkBζ in B virus-infected cell nuclei. HFFs were infected with B virus at m.o.i. of 10 for 1, 2, 3, 4, 5 and 6 h. Cells were stained with anti-IκBζ antibody (green) and nuclei were stained with Hoechst 33342 (red).

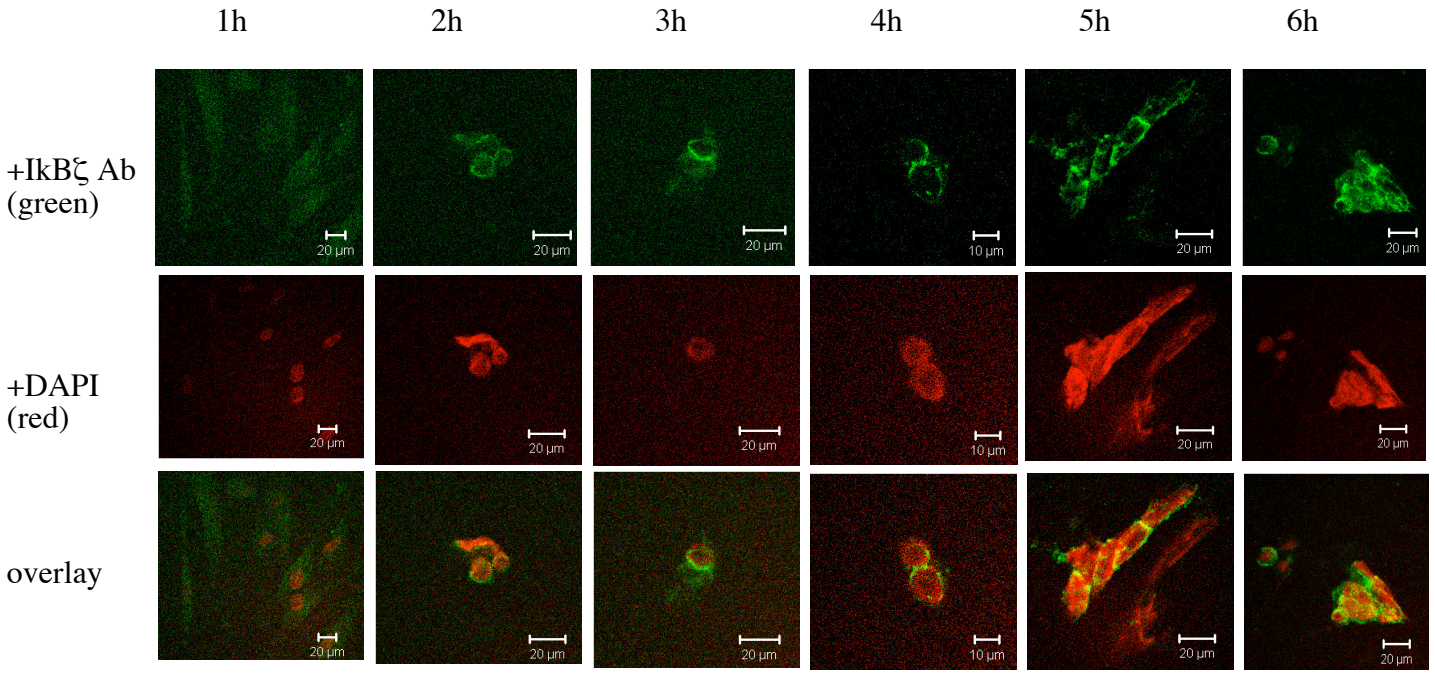

Fig 25. Immunofluorescent staining of IkBζ in HSV-1 infected cell nuclei. HFFs were infected with HSV-1 at m.o.i. of 10 for 1, 2, 3, 4, 5 and 6 h. Cells were stained with anti-IκBζ antibody (green) and nuclei were stained with Hoechst 33342 (red).

receptors) and HDAC5 (histone deacetylase 5) in the nucleus. Because not every cell was observed to be IκBζ positive, cells were probably not evenly infected, or IκBζ protein levels were too low to be detected in some cells.

Expression of NF-κB target genes in B virus infected IκBζ knockdown cells. IκBζ had been identified as a NF-κB inhibitor and is bound preferentially to NF-κB p50 (155) (169) (171). *In vitro* experiments indicated that the IκBζ carboxyl-terminal domain with the ankyrin-repeats inhibits the DNA binding of the p65·p50 heterodimer as well as the p50 homodimer (171). To investigate whether one role of IκBζ in B virus infected cells is to negatively regulate NF-κB activity in the nucleus, experiments were designed to measure NF-κB DNA binding activity under conditions of knockdown and restoration of IκBζ during virus infection. RNA interference was used to knockdown the IκBζ gene. The knockdown rates of IκBζ gene for samples collected from each time point were shown in Table 5, and the average knockdown rate was 71.7%. The NF-κB transactivated target gene CCL2 had mRNA levels elevated at 4 h and 5 h post B virus infection, whereas CXCL1 and CXCL3 mRNA levels were elevated at both 4 h and 6 h in IκBζ knockdown cells (Fig 26). These data suggest that B virus inducible IκBζ correlated with inhibition of expression of the NF-κB regulated cytokine/chemokine gene CCL2, CXCL1, and CXCL3, and may be causally related. Other NF-κB transactivated target genes, IL-6, CXCL2, and IL-8, however, showed repressed mRNA levels post B virus infection in IκBζ knockdown HFF cells, which suggested that IκBζ-NF-κB complex is not the only factor regulating these cytokine genes in B virus infected cells.

Table 5. The average IκBζ knockdown rate of samples collected from each time point. (n=4). The expression level of IκBζ were quantified by real-time RT-PCR and the percent of IκBζ knockdown rate was determined by comparing the IκBζ expression level between B virus infected IκBζ siRNA-treated cells and B virus infected negative siRNA-treated cells.

Hour post infection	1 h	2 h	3 h	4 h	5 h	6 h
IκBζ knockdown rate	80%	74%	56%	61%	71%	88%

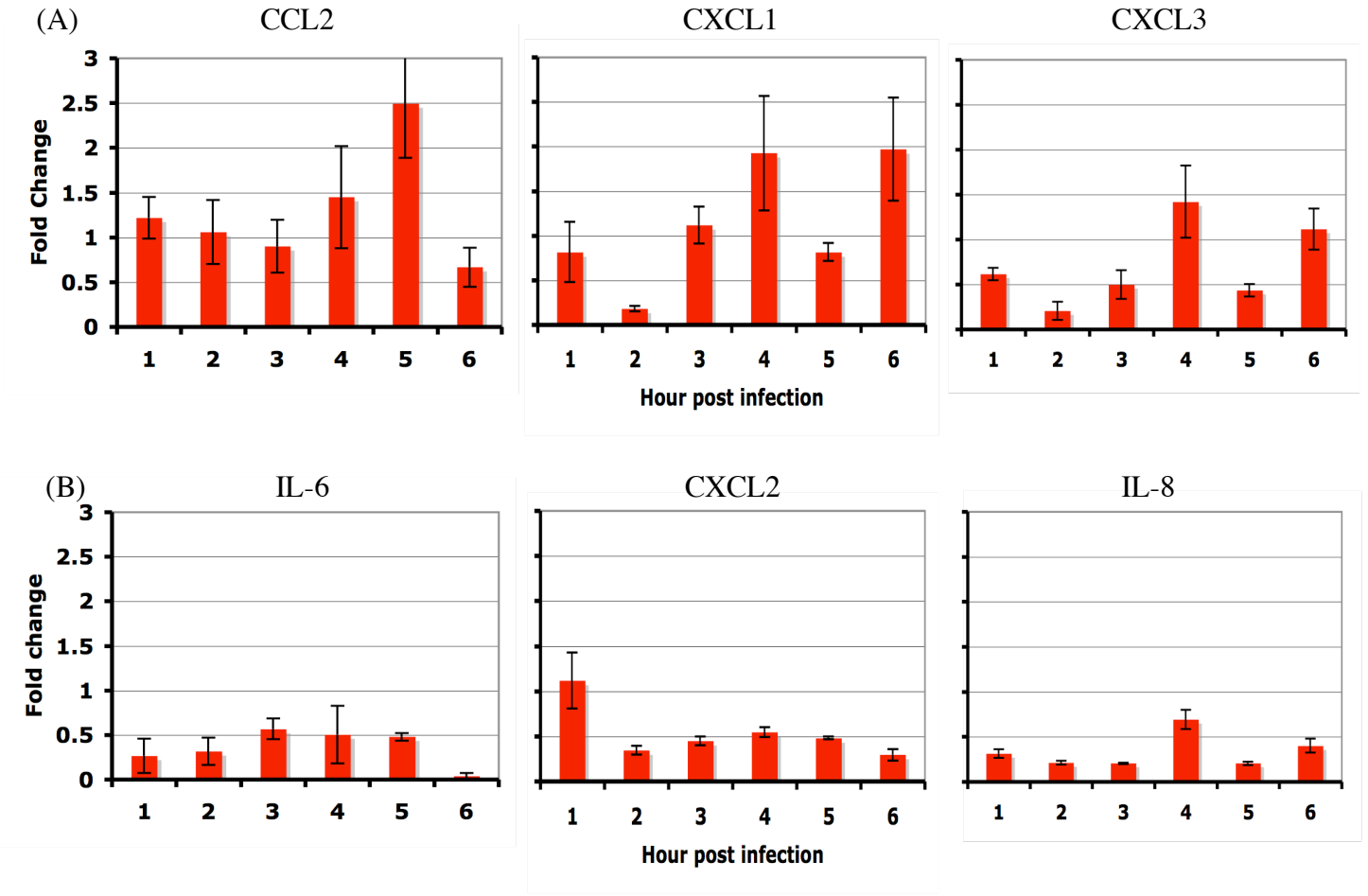

Fig 26. Cytokine gene expressions in B virus infected IκBζ gene knockdown HFF cells. Gene expression levels in B virus infected IκBζ gene knockdown cells were quantified by real-time RT-PCR and compared to B virus infected negative control siRNA-treated cells. (A) IκBζ knockdown enhances some cytokine genes. (B) Cytokine genes that are not enhance by IκBζ. Data are the means ± standard deviations (n = 4).

SECTION IV. DISCUSSION

Human cell culture model system of B virus infection

B virus infection frequently results in deadly outcomes in foreign host systems. The reasons underlying the severe morbidity and mortality are poorly understood. B virus has co-evolved and adapted to have a productive relationship with macaque hosts, but encountering human hosts far less frequently has left both without a strategy for peaceful co-existence. As a result, human hosts suffer severe morbidity and frequent mortality as a result of B virus zoonotic infection. Primary human foreskin fibroblasts, one type of target cell and representative of those infected *in vivo*, were selected for these studies. Additionally, primary HFF cells in culture have regulated cell cycle machinery and become contact-inhibited when grown to confluence, mimicking the quiescent state of the dermis. As such, cultures of HFF cells infected with B virus were used as a model of foreign host cells to study the molecular events at early time points after B virus infection. To design a natural host infection model, the same cell type, HFF, was infected with HSV-1, because human cells are also the natural hosts to HSV-1 infection.

Comparative analysis of virus-induced, global cellular transcriptional remodeling provided opportunities to examine previously published data on host gene expression in different cell types induced by different strains of HSV-1, as well as, for the first time, examine the events resulting from B virus infection (151) (111) (79) (129). Using growth-arrested cells for analysis of virus-induced host cell expression remodeling

generally reduces the variability of transcriptional activity, because cells are spread across the cell cycle and therefore represent the broadest spectrum of metabolic activities. The cell line used in these studies was asynchronous prior to virus inoculation. By using high m.o.i. (i.e. 10) to cause near simultaneous infection of all cells at the initial round of viral addition, the average behavior of all infected cells was measured. The replication kinetics of B virus proved faster, although not significantly faster, than that of HSV-1 replication in HFFs (Fig 1).

Using HFF cell cultures to serve as a model in which the events of host cell responses are mapped provides identification of potential targets for blocking or modulating pathogenesis to ultimately reduce morbidity, the establishment of latency, or virus reactivation. The field of virus-host interactions has progressed greatly in recent years, particularly with the help of more user-friendly bioinformatics tools as rich mines of data are gathered. Data obtained from cell lines, integrated with data from animal model studies, will ultimately lead to further understanding of B virus infection outcome in non-adapted foreign host cells.

Adapted cell model system of B virus infection: macaque cells in culture

B virus is indigenous in macaque monkeys, and disease in natural hosts, macaques, is often mild or inapparent. Are there differences in early innate host responses when B virus infects macaques vs. humans? From the results of B virus

infection in human foreskin fibroblast cells, defective induction of innate immune response genes was observed, suggesting that B virus is not able to induce innate immune responses in human cells. Needless to say there are many limitations inherent in a cell culture model system when the ultimate goal is to understand B virus infection in humans, however, the virus dynamics within human cells in cultures provides a mechanism to examine molecular events of infection in a controlled microenvironment. Parallel to this B virus-infected human cell culture model, future studies with a B virus-infected monkey cell culture model will provide a mechanism by which comparative analysis of the early cellular responses in cells from the natural host versus the foreign host can be performed. Preliminary microarray data from B virus-infected monkey cells (MK-2, rhesus monkey kidney cell) showed that immune response genes, including IFN-α4, IFN-α5, IL-6 (IFN-β2), IRF1, IL-16, IL24, CD24, PLUNC, TNSF9, TRAF3IP2, MAP3K, ATF3, JUB, NR4A2, SCAP1, FGFR1 etc., were induced post 1 hour B virus infection at 37°C. Studies on the activation status of STAT-1 phosphorylation, which plays a role to regulate IRF-1 in IFN pathway, also demonstrated that B virus triggers IFN responses in the natural host cells, MK-2. The impact of these observations must be elucidated with future studies, but these preliminary results *in vitro* using macaque cells in culture a model system to study host responses to B virus infection. Currently, the data presented in this dissertation, however, strongly support that B virus infection in macaque cells result in induction of comparable cytokine responses to those seen in human cells infected with HSV-1. B virus in foreign host cells, however, results in no cytokine responses, suggesting that B virus effectively blocks the foreign host cytokine responses.

The implication is that the adaptation of macaques to B virus infection is similar to the adaptation of humans to HSV-1. The absence of these adaptive responses correlates with the absence of a neutrophil-based inflammatory response. Whether these observations correlate with the dramatic morbidity and mortality of B virus in foreign hosts requires many more studies, but forms the foundation for interesting speculation and the examination of a number of hypotheses.

Microarray tools: snapshots in time of virus: host cell interactions

DNA microarray technology is a powerful tool to study the simultaneous transcriptional expression of thousands of genes. The gene chips (human genome U133 set, Affymetrix) that were used for these studies contained 45,000 probe sets representing more than 39,000 transcripts derived from ~33,000 well-documented human genes. Nevertheless, processing and mining the large amount of data generated by this high throughput technology are challenging, underscoring the need for statistical analysis and biological interpretation algorithms to determine what constitutes statistically significant, virus-specific host expression remodeling snapshots at time points following virus infection.

In the microarray experimental design, an important issue is to determine how many replicates are optimal for statistical analysis. Power analyses often indicate that larger sample sizes increase sensitivity and specificity. Small samples sizes (two independent replicates) per condition (time and virus) in the experimental design of these

investigations coupled with efforts to maintain low FDR q value and ANOVA p value can result in low power to detect differential expression. These microarray data, however were used to gather the overall gene expression profile and to provide a snapshot of which genes were activated in the early stages of B virus infection in human cells in culture. Compensation to balance the low power problem was accomplished by obtaining an extensive list of genes with the lowest 1000 p- and q-value to be sorted for differentially expressed genes. Since microarrays were considered as semiquantitative assays, any gene expression levels of interest were more precisely analyzed using real-time RT-PCR.

In microarray experiments, another inherent problem is that the expression levels of non-coding genes, such as microRNAs (miRNAs) and siRNAs, were not detectable on gene chips. In these experiments, although it was not clear whether there are any miRNA or siRNA sequences in B virus genome, at least one miRNA out of the predicted 13 miRNA precursors were identified, while in HSV-1 genomes there have been 24 miRNA candidates (32) that may play important roles in redirecting viral defense functions of host cells (84). The discoveries of miRNAs encoded by other herpesvirus family, such as Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus, mouse gammaherpesvirus 68, and human cytomegalovirus (125) (140) (124), also strongly suggest that B virus has likely undergone parallel selection that enables manipulation of host genes involved with antiviral defenses.

Kinetic analysis of microarray data: observations of dynamic virus: host interactions

In the microarray studies presented in this dissertation, early host cellular responses induced by two closely neuroinvasive alphaherpesviruses, B virus and HSV-1, were comparatively analyzed in a common cell type, human foreskin fibroblasts, at different time points during the first 5 hours of infection, including at the time of virus attachment to cells. Although these cells showed almost no measurable changes during virus attachment of either B virus or HSV-1, there was one gene, IL-6ST, induced by each virus. This gene encodes the transducer chain shared by many cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF). All of these cytokines act via a receptor complex in which signaling is triggered by homodimerization or heterodimerization with gp130. Downstream of this cytokine-IL-6ST pathway, a variety of biochemical events take place, including activation of cytoplasmic tyrosine kinases that are associated with IL-6ST and modification of transcription factors. To monitor whether virus attachment induces any host gene expression, an alternative experimental design to performing virus adsorption at 4°C, preventing virus penetration, U.V. inactivated virus can be utilized in future studies.

The observation that increased expression of only three genes occurred at 3 h p.i. and 31 genes at 5 h p.i., decreased expression of 27 genes at 3 h p.i. and 742 genes at 5 h p.i. that overlapped between B virus and HSV-1 infected cells (Fig 3) demonstrated that

cellular gene expression patterns began to be remodeled differentially between B virus and HSV-1 shortly after viruses entered into cells. Although most host genes expressed following B virus and HSV-1 infection were different, the majority of them can still be grouped according to similar functions, e.g., gene expression, cell-to-cell signaling and interaction, cellular functions, nervous system development and function, and tissue morphology (data not shown).

B virus appeared to down-regulate fewer host genes than HSV-1 (Fig 2). It is not clear why human host cells respond to these two similar simplexviruses so differently with regard down-regulation of host genes. Alphaherpesviruses encode a structural protein, the virion host shutoff (*vhs*) protein, which stimulates nonspecific degradation of cellular mRNA, inhibiting host cell protein synthesis during the early virus infection. Perhaps B virus vhs function is not as efficient as HSV-1, or the human host cell RNAs are more resistant to B virus vhs degradation. Another possibility is that B virus does not activate transcriptional repressors as HSV-1 does to shut down host cell gene transcription in human cells.

The lack of innate immune responses in B virus infected cells: stealth virus in the environment of the foreign host cell.

To differentiate early host innate responses following B virus or HSV-1 infection in human cells, innate immune response genes were sorted from the microarray data to

run hierarchical cluster analysis. The studies described in this dissertation show for the first time that B virus infection results in the expression of almost no innate immunity related genes, e.g., to the IFN signaling pathway, cytokine or chemokine production. On the other hand, HSV-1 infection of human cells was followed by host expression of CXCL1, CXCL2, CXCL3, CXCL6, CXCL8 (IL-8), CCL2, IL-1β, IL-6, IL-11, IRF-1, IFNAR2 and SOCS3 etc., which are involved in chemotaxis, inflammation, and immune cell proliferation (Fig 7 & 8). The observations in HSV-1-infected cells agreed with other published microarray data (151) showing that HSV-1 infection results in a strong host cell responses in the natural host cells, but this study also demonstrates for the first time that host cells infected with B virus appear, within the first five hours, not to respond as if infected. The implication is that B virus can mask its presence in human cells, especially in the early stages of infection, allowing B virus to proceed unimpeded by the innate defenses of the target cell. How this facilitates the dramatic pathogenesis of the virus in foreign hosts will certainly be the topic of many future studies. In contrast, host cells infected with HSV-1 responded measurably to infection, and in turn HSV-1 induced suppressor of cytokine signaling-3 (SOCS3), a host cell negative regulator of the JAK-SATA pathway to inhibit antiviral gene expression (Fig 7) (172). These events reflect the orchestrated events within in host cell that has adapted to a specific virus infection.

Sequence of events from cytoplasm to nucleus during the course of virus infection (Virus-specific regulation of the host cell NF-κB pathway)

Regardless of the differences in host gene remodeling following each virus infection, nalyses of microarray data revealed that shortly after the onset of infection, NF-κB signaling pathways were engaged following either virus infection in human cells, i.e., p50 was translocated to the nucleus and bound to the canonical p65/p50 promoter to drive the expression of NF-κB target genes. The stimuli that triggered these events following B virus infection are unknown, but investigators have shown that HSV-1 can utilize TLR2, TLR9, gD-HveA binding or the expression of ICP4 and ICP27 to activate NF-κB (110) (83) (98, 122). If the NF-κB, p65/p50 translocation is seen in either virus infection of human cells, what causes the differential activation of cytokine transcription? Recent identification of a negative regulator of p50 binding activity in the nucleus, IκBζ, was evaluated. Examination of each virus infection revealed that there was differential regulation of the gene encoding IκBζ, thus this candidate protein was investigated as part of these studies.

To further understand the role of IκBζ as a negative regulator of NF-κB p50 blocking NF-κB DNA binding activity (155) (169) (171), IκBζ was silenced. This resulted in the induction of the expression of a set of cytokine and chemokine genes, including CCL2, CXCL1, and CXCL3, in B virus infected humans cells, making IκBζ a candidate inhibitor of host innate response genes. Other genes, however, including IL-6, CXCL2, Il-8 were not restored upon silencing the IκBζ gene in the B virus infected

HFFs. Either the remaining IκBζ activity (knockdown rate was 71%) was able to block binding of p65/p50 transactivation of cytokines, or an IκBζ-independent mechanism exists to regulate specific cytokine genes. In IκBζ knockout mice, investigators have shown that IκBζ is involved in NF-κB dependent activation of IL-6 by functioning as a coactivator (169). It is therefore intriguing to speculate that B virus induced IκBζ functions not only as an inhibitor, but also as a coactivator to regulate NF-κB dependent genes expression. Due to the various promoter sequences specific in different NF-κB target genes, the role of IκBζ in regulating transcriptional activity of each NF-κB target genes is likely gene-specific.

Since HSV-1 infection also resulted in IκBζ induction in HFF cells, these studies evaluated other regulators of cytokine genes. Besides IκBζ, NF-κB negative regulators IκBα (NFKBIA), IkBε (NFKBIE), and BCL-3 were found to be induced in HSV-1 infected cells. This feedback inhibition presumably is destined to restore the inhibited NF-κB state, thereby, controlling the NF-κB regulated genes to block apoptotic functions. Meanwhile, many other NFκB genes including p100 (p52 precursor), p105 (p50 precursor), REL, and RelB were all induced by HSV-1, but not by B virus. Induced expression of the NF-κB genes is thought to replenish NF-κB in the nuclei to compensate for the activity of NF-κB blocked by IκBζ. This may be one explanation for the observation that expression of NF-κB regulated innate immune response genes were not inhibited in HSV-1 infected human cells.

Another factor that may play a role in HSV-1 infected cells is the cytokine IL-1β, which can activate the NF-κB signaling pathway. This results in expression of various inflammation- and innate immunity-related genes that continuously up-regulate the induction of cytokines or chemokines. Although IL-1β was not induced in microarray data, real-time RT-PCR data showed that IL-1β was highly induced (> 6.9- to 31-fold) by HSV-1. This discrepancy may be because that IL-1β gene has splice variants and the cDNA probe sets on the Affymetrix chip can not detect IL-1β well. On the other hand, no IL-1β was found to be induced in B virus-infected cells either by microarray or real-time RT-PCR analysis. The lack of IL-1β induction in B virus-infected cells may contribute to the lack of cytokine and chemokine induction.

Neutrophil chemotaxis during virus infection provides inflammatory responses that dampen virus replication.

Based on the microarray data and neutrophil transmigration result shown in Fig. 8, HFFs secreted cytokines and chemokines, e.g., IL-6, IL-8, CXCL1, CXCL2, CXCL3 and CCL2 to induce neutrophil chemotaxis after HSV-1 infection, but not after B virus infection. This suggests that in our *in vitro* HSV-1 natural host cells model, innate immune response system includes infiltration of inflammatory cells into the virus infected sites. There was a basal level of chemoattractant activity found in mock-infected cell supernatants leading to neutrophil transmigration. It has been reported that

mitochondrial derived peptides, e.g., mitochondrial encoded NADH dehydrogenase subunit 1 (ND1), are potent chemotactic ligands that initiate neutrophil signaling pathways (146). Therefore, it is possible that the release of these mitochondrial derived peptides from disintegrating mitochondria is responsible for the observation that mock-infected cell supernatants had low levels of chemoattractant activity.

The foreign host cell model for B virus infection revealed that infected HFFs lacked the abilities to produce chemoattractant cytokines or chemokines. This is intriguing because this suggests that B virus is able to escape host immune response surveillance at early time points following infection. The induction of IκBζ to block NF-κB dependent transcriptional activity may explain why there is no chemoattractant cytokine or chemokine production post B virus infection. Experimental evidence did not rule out the possibility that B virus also produced some negative chemoattractant regulator to block neutrophil transmigration, since mock-infected cell supernatants were found to have greater chemoattractant activity than B virus-infected cell supernatant. To further confirm the neutrophil chemoattractant inhibitory effect in B virus-infected HFFs supernatants was due to an unidentified inhibitor(s) produced by B virus, strong neutrophil chemoattractants, IL-8 and f-MLP, were added to B virus-infected HFF supernatant separately to perform neutrophil transmigration assay. The results indicated that B virus inhibited both IL-8 and fMLP-mediated neutrophils transmigration. Interestingly, mock-infected cell supernatants inhibited both IL-8 and fMLP-mediated neutrophil transmigration rates as well. It is still unknown what chemotactic inhibitor(s) are produced by fibroblasts under the conditions of B virus infection or mock-infection.

But fibroblasts might be prompted to produce chemotactic inhibitors as a result of stress due to growth in serum-free medium. HSV-1 infected cell supernatants reduced chemotaxis relative to controls, but significantly less effectively than B virus inhibition of IL-8-mediated neutrophil transmigration (Fig 15). These observations may be explained by the observations that HSV-1 infected cells derived chemoattractants desensitized G protein coupled chemoattractant receptors on the cell surfaces. On the other hand, the addition of fMLP to HSV-1 infected cell supernatants did not inhibit neutrophil transmigration in later time points (i.e. 5 h p.i.) (Fig 16), implying that fMLP-mediated neutrophil migration signaling pathway was not affected and remained functional.

Neutrophil infiltration has been observed in HSV-1 infection in keratinocytes and corneal epithelial cells (102) (156) (106). Severe HSV-1 infections are observed in corneas and footpads of mice depleted of neutrophils (156) (160). It has also been shown that there is a high incidence of HSV-1 infection in patients rendered neutropenic as a result of chemotherapy (8) (78). Neutrophils mediated protection against HSV-1 via several mechanisms, including antibody-dependent cellular cytotoxicity, Fc-mediated phagocytosis, complement dependent chemotaxis and phagocytosis. Neutrophil can directly inactivate HSV-1 by realeasing defensin or reactive oxygen species (54) (168). Neutrophils may also exert their antiviral effects indirectly by producing chemokines or cytokines, which in turn recruit other effector cells, such as T cells, macrophages and NK cells to the site of virus infection to participate in nonspecific or specific antiviral defense mechanisms (25). The data presented in this dissertation showed that HFFs did not respond to B virus infection by inducing any chemokines or cytokines with

chemoattractant activity, while HFFs responded to HSV-1 and induced neutrophil chemotaxis. These data support that neutrophils play an important role in the innate immunity/inflammatory to control/restrict virus invasion of adjacent c natural host cells, but not in the environment of foreign host cells.

Conclusion

Overall, the study of early cell signaling events following B virus and HSV-1 infection provides the foundation for future studies to unravel the subsequent cellular events that can provide a better understanding of what causes different disease outcomes as a result of the same virus infection in different host systems. HSV-1 infection not only induces innate immune responsive genes in human cells, but it also has mechanisms for overcoming the host defense system. This likely reflects the evolution of HSV-1 with human cells, resulting in the virus' capability to establish life-long latent infection. B virus, however, utilizes IκBζ as a direct way to block the induction of NF-κB target genes, thereby inhibiting human host innate immune responses. Ultimately, this may lead to the inability of B virus to coexist within human hosts. Because innate immunity is critical for the generation of effective adaptive immune responses, the block of NF-κB may prove to be a major correlate in the severe morbidity and mortality resulting from human B virus infection. Ultimately, this dissertation provided the opportunities to investigate the differences in virus behavior within natural and foreign host microenvironments, paving the way for utilizing this system to identify critical targets of B virus infection. Identification of these targets furthers the understanding of how

emerging and re-emerging viruses may behave in foreign hosts, and additionally provides invaluable information for control of virus infections in foreign versus natural hosts.

REFERENCE

1. **Allison, D. B., X. Cui, G. P. Page, and M. Sabripour.** 2006. Microarray data analysis: from disarray to consolidation and consensus. Nat Rev Genet **7:**55-65.
2. **Amici, C., G. Belardo, A. Rossi, and M. G. Santoro.** 2001. Activation of I kappa b kinase by herpes simplex virus type 1. A novel target for anti-herpetic therapy. J Biol Chem **276:**28759-66.
3. **Ankel, H., D. F. Westra, S. Welling-Wester, and P. Lebon.** 1998. Induction of interferon-alpha by glycoprotein D of herpes simplex virus: a possible role of chemokine receptors. Virology **251:**317-26.
4. **Arase, H., N. Arase, and T. Saito.** 1995. Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exp Med **181:**1235-8.
5. **Artenstein, A. W., C. B. Hicks, B. S. Goodwin, Jr., and J. K. Hilliard.** 1991. Human infection with B virus following a needlestick injury. Rev Infect Dis **13:**288-91.
6. **Baldwin, A. S., Jr.** 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol **14:**649-83.
7. **Barchet, W., M. Cella, B. Odermatt, C. Asselin-Paturel, M. Colonna, and U. Kalinke.** 2002. Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. J Exp Med **195:**507-16.
8. **Beattie, G., J. Whelan, J. Cassidy, L. Milne, S. Burns, and R. Leonard.** 1989. Herpes simplex virus, Candida albicans and mouth ulcers in neutropenic patients with non-haematological malignancy. Cancer Chemother Pharmacol **25:**75-6.
9. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. Genes Dev **6:**1899-913.
10. **Benjamini, Y., and Hochberg, Y.** 1995. Controlling the false discovery rate – A practical and powerful approach to multiple testing. Roy Stat Soc B **57:**289-300.
11. **Benson, P. M., S. L. Malane, R. Banks, C. B. Hicks, and J. Hilliard.** 1989. B virus (Herpesvirus simiae) and human infection. Arch Dermatol **125:**1247-8.
12. **Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather.** 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol **17:**189-220.
13. **Bishop, G. A., S. D. Marlin, S. A. Schwartz, and J. C. Glorioso.** 1984. Human natural killer cell recognition of herpes simplex virus type 1 glycoproteins: specificity analysis with the use of monoclonal antibodies and antigenic variants. J Immunol **133:**2206-14.
14. **Blank, V., P. Kourilsky, and A. Israel.** 1992. NF-kappa B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats. Trends Biochem Sci **17:**135-40.

15. **Bolstad, B. M., R. A. Irizarry, M. Astrand, and T. P. Speed.** 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics **19:**185-93.
16. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R. P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-kappa B participates in transactivation through a kappa B site. Mol Cell Biol **12:**685-95.
17. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a kappa B DNA-binding protein with homology to the rel oncogene and to cell-cycle motifs. Nature **348:**76-80.
18. **Breen, G., Lamb, SG, Otaki, AT.** 1958. Monkey-bite encephalomyelitis: report of a case with recovery. Brit Med J **2:**22-3.
19. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. Proc Natl Acad Sci U S A **90:**2532-6.
20. **Bryan, B. L., C. D. Espana, R. W. Emmons, N. Vijayan, and P. D. Hoeprich.** 1975. Recovery from encephalomyelitis caused by Herpesvirus simiae. Report of a case. Arch Intern Med **135:**868-70.
21. **Budhraja, V., E. Spitznagel, W. T. Schaiff, and Y. Sadovsky.** 2003. Incorporation of gene-specific variability improves expression analysis using high-density DNA microarrays. BMC Biol **1:**1.
22. **Bull, P., T. Hunter, and I. M. Verma.** 1989. Transcriptional induction of the murine c-rel gene with serum and phorbol-12-myristate-13-acetate in fibroblasts. Mol Cell Biol **9:**5239-43.
23. **Burnet, F. M., Lush, D., Jackson, A.V.** 1939. The propagation of herpes B and pesudorabies viruses on the chorioallantois. Aust J Exp Biol Med Sci **17:**35-52.
24. **Campbell, J. J., E. F. Foxman, and E. C. Butcher.** 1997. Chemoattractant receptor cross talk as a regulatory mechanism in leukocyte adhesion and migration. Eur J Immunol **27:**2571-8.
25. **Cassatella, M. A.** 1995. The production of cytokines by polymorphonuclear neutrophils. Immunol Today **16:**21-6.
26. **Chee, A. V., P. Lopez, P. P. Pandolfi, and B. Roizman.** 2003. Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. J Virol **77:**7101-5.
27. **Chettibi, S. a. F., M.W.J. .** 1999. Inflammation: Basic Principles and Clinical Correlates. (eds Gallin JI and Snyderman R). (Lipincott, Williams and Wilkinson, Philadelphia)**:**865-881.
28. **Cicchetti, G., P. G. Allen, and M. Glogauer.** 2002. Chemotactic signaling pathways in neutrophils: from receptor to actin assembly. Crit Rev Oral Biol Med **13:**220-8.
29. **Clapham, D. E., and E. J. Neer.** 1993. New roles for G-protein beta gamma-dimers in transmembrane signalling. Nature **365:**403-6.

30. **Cohen, J. I., D. S. Davenport, J. A. Stewart, S. Deitchman, J. K. Hilliard, and L. E. Chapman.** 2002. Recommendations for prevention of and therapy for exposure to B virus (cercopithecine herpesvirus 1). Clin Infect Dis **35:**1191-203.
31. **Cohney, S. J., D. Sanden, N. A. Cacalano, A. Yoshimura, A. Mui, T. S. Migone, and J. A. Johnston.** 1999. SOCS-3 is tyrosine phosphorylated in response to interleukin-2 and suppresses STAT5 phosphorylation and lymphocyte proliferation. Mol Cell Biol **19:**4980-8.
32. **Cui, C., A. Griffiths, G. Li, L. M. Silva, M. F. Kramer, T. Gaasterland, X. J. Wang, and D. M. Coen.** 2006. Prediction and identification of herpes simplex virus 1-encoded microRNAs. J Virol **80:**5499-508.
33. **Cunningham, A. L., and Z. Mikloska.** 2001. The Holy Grail: immune control of human herpes simplex virus infection and disease. Herpes **8 Suppl 1:**6A-10A.
34. **Daniel, M. D., F. G. Garcia, L. V. Melendez, R. D. Hunt, J. O'Connor, and D. Silva.** 1975. Multiple Herpesvirus simiae isolation from a rhesus monkey which died of cerebral infarction. Lab Anim Sci **25:**303-8.
35. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415-21.
36. **Davenport, D. S., D. R. Johnson, G. P. Holmes, D. A. Jewett, S. C. Ross, and J. K. Hilliard.** 1994. Diagnosis and management of human B virus (Herpesvirus simiae) infections in Michigan. Clin Infect Dis **19:**33-41.
37. **Davidson, W. L., and K. Hummeler.** 1960. B virus infection in man. Ann N Y Acad Sci **85:**970-9.
38. **de Martin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F. H. Bach.** 1993. Cytokine-inducible expression in endothelial cells of an I kappa B alpha-like gene is regulated by NF kappa B. Embo J **12:**2773-9.
39. **de Veer, M. J., M. Holko, M. Frevel, E. Walker, S. Der, J. M. Paranjape, R. H. Silverman, and B. R. Williams.** 2001. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol **69:**912-20.
40. **Dennis D Taub, E. S.** 2000. Biological Responses to Chemokine Superfamily Members. Current protocols in Immunology**:**6.12.1-6.12.32.
41. **Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman.** 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A **95:**15623-8.
42. **Diaz-Meco, M. T., I. Dominguez, L. Sanz, P. Dent, J. Lozano, M. M. Municio, E. Berra, R. T. Hay, T. W. Sturgill, and J. Moscat.** 1994. zeta PKC induces phosphorylation and inactivation of I kappa B-alpha in vitro. Embo J **13:**2842-8.
43. **Eberle, R., D. Black, and J. K. Hilliard.** 1989. Relatedness of glycoproteins expressed on the surface of simian herpes-virus virions and infected cells to specific HSV glycoproteins. Arch Virol **109:**233-52.
44. **Eberle, R., and J. Hilliard.** 1995. The simian herpesviruses. Infect Agents Dis **4:**55-70.
45. **Espana, C.** 1973. Herpesvirus simiae infection in Macaca radiata. Am J Phys Anthropol **38:**447-54.

46. **Eto, A., T. Muta, S. Yamazaki, and K. Takeshige.** 2003. Essential roles for NF-kappa B and a Toll/IL-1 receptor domain-specific signal(s) in the induction of I kappa B-zeta. Biochem Biophys Res Commun **301:**495-501.
47. **Fierer, J., P. Bazely, and A. I. Braude.** 1973. Herpes B virus encephalomyelitis presenting as ophthalmic zoster. A possible latent infection reactivated. Ann Intern Med **79:**225-8.
48. **Fitzgerald-Bocarsly, P., D. M. Howell, L. Pettera, S. Tehrani, and C. Lopez.** 1991. Immediate-early gene expression is sufficient for induction of natural killer cell-mediated lysis of herpes simplex virus type 1-infected fibroblasts. J Virol **65:**3151-60.
49. **Franzoso, G., V. Bours, V. Azarenko, S. Park, M. Tomita-Yamaguchi, T. Kanno, K. Brown, and U. Siebenlist.** 1993. The oncoprotein Bcl-3 can facilitate NF-kappa B-mediated transactivation by removing inhibiting p50 homodimers from select kappa B sites. Embo J **12:**3893-901.
50. **Franzoso, G., V. Bours, S. Park, M. Tomita-Yamaguchi, K. Kelly, and U. Siebenlist.** 1992. The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-kappa B-mediated inhibition. Nature **359:**339-42.
51. **Freifeld, A. G., J. Hilliard, J. Southers, M. Murray, B. Savarese, J. M. Schmitt, and S. E. Straus.** 1995. A controlled seroprevalence survey of primate handlers for evidence of asymptomatic herpes B virus infection. J Infect Dis **171:**1031-4.
52. **Galdiero, S., M. Vitiello, M. D'Isanto, E. Di Niola, L. Peluso, K. Raieta, C. Pedone, M. Galdiero, and E. Benedetti.** 2004. Induction of signaling pathways by herpes simplex virus type 1 through glycoprotein H peptides. Biopolymers **76:**494-502.
53. **Ganchi, P. A., S. C. Sun, W. C. Greene, and D. W. Ballard.** 1992. I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. Mol Biol Cell **3:**1339-52.
54. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer.** 1985. Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest **76:**1427-35.
55. **Gay, F. P., Holden, M. .** 1933. The herpes encephalitis problem. J Infect Dis **53:**287-303.
56. **Ghanekar, S., L. Zheng, A. Logar, J. Navratil, L. Borowski, P. Gupta, and C. Rinaldo.** 1996. Cytokine expression by human peripheral blood dendritic cells stimulated in vitro with HIV-1 and herpes simplex virus. J Immunol **157:**4028-36.
57. **Ghosh, S., M. J. May, and E. B. Kopp.** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol **16:**225-60.
58. **Gilmore, T. D.** 2006. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene **25:**6680-4.

59. **Goodkin, M. L., A. T. Ting, and J. A. Blaho.** 2003. NF-kappaB is required for apoptosis prevention during herpes simplex virus type 1 infection. J Virol **77:**7261-80.
60. **Halford, W. P., B. M. Gebhardt, and D. J. Carr.** 1996. Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. J Immunol **157:**3542-9.
61. **Hayashi, T., T. Sekine, and T. Okamoto.** 1993. Identification of a new serine kinase that activates NF kappa B by direct phosphorylation. J Biol Chem **268:**26790-5.
62. **He, B., M. Gross, and B. Roizman.** 1997. The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. Proc Natl Acad Sci U S A **94:**843-8.
63. **He, J., H. Ichimura, T. Iida, M. Minami, K. Kobayashi, M. Kita, C. Sotozono, Y. I. Tagawa, Y. Iwakura, and J. Imanishi.** 1999. Kinetics of cytokine production in the cornea and trigeminal ganglion of C57BL/6 mice after corneal HSV-1 infection. J Interferon Cytokine Res **19:**609-15.
64. **Heit, B., S. Tavener, E. Raharjo, and P. Kubes.** 2002. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J Cell Biol **159:**91-102.
65. **Henkel, T., U. Zabel, K. van Zee, J. M. Muller, E. Fanning, and P. A. Baeuerle.** 1992. Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit. Cell **68:**1121-33.
66. **Hilliard, J. K., D. Black, and R. Eberle.** 1989. Simian alphaherpesviruses and their relation to the human herpes simplex viruses. Arch Virol **109:**83-102.
67. **Hilliard, J. K., R. Eberle, S. L. Lipper, R. M. Munoz, and S. A. Weiss.** 1987. Herpesvirus simiae (B virus): replication of the virus and identification of viral polypeptides in infected cells. Arch Virol **93:**185-98.
68. **Hilton, M. J., D. Mounghane, T. McLean, N. V. Contractor, J. O'Neil, K. Carpenter, and S. L. Bachenheimer.** 1995. Induction by herpes simplex virus of free and heteromeric forms of E2F transcription factor. Virology **213:**624-38.
69. **Holmes, G. P., L. E. Chapman, J. A. Stewart, S. E. Straus, J. K. Hilliard, and D. S. Davenport.** 1995. Guidelines for the prevention and treatment of B-virus infections in exposed persons. The B virus Working Group. Clin Infect Dis **20:**421-39.
70. **Holmes, G. P., J. K. Hilliard, K. C. Klontz, A. H. Rupert, C. M. Schindler, E. Parrish, D. G. Griffin, G. S. Ward, N. D. Bernstein, T. W. Bean, and et al.** 1990. B virus (Herpesvirus simiae) infection in humans: epidemiologic investigation of a cluster. Ann Intern Med **112:**833-9.
71. **Huang, A. S., and R. R. Wagner.** 1964. Penetration of Herpes Simplex Virus into Human Epidermoid Cells. Proc Soc Exp Biol Med **116:**863-9.

72. **Hull, R. N.** 1973. The simian herpesviruses. In: Kaplan AS, ed. The herpesviruses. New York: Academic Press.**:**389-425.
73. **Hummeler, K., Davidson, W.T.,Henle W, , and A. LaBoccetta, Ruch HG.** 1959. Encephalomyelitis due to infection with Herpesvirus simiae (herpes B virus): a report of two fatal laboratory-acquired cases. N Engl J Med **261:**64-8.
74. **Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249-64.
75. **Isaaks, A., and Lindenman, J.** 1957. Virus interference. I. The interferon. Proc. R. Soc. London B. **147:**258-267.
76. **Janeway, C. A., Travers, P., Walport, M., and Shlomchik,M.** 2005. Adaptive immunity to infection. In "Immunobiology: the immune system in health and disease" 6th ed. Garland, New York.
77. **Jang, K. L., B. Pulverer, J. R. Woodgett, and D. S. Latchman.** 1991. Activation of the cellular transcription factor AP-1 in herpes simplex virus infected cells is dependent on the viral immediate-early protein ICPO. Nucleic Acids Res **19:**4879-83.
78. **Janmohamed, R., J. E. Morton, D. W. Milligan, M. J. Leyland, and B. Coupland.** 1990. Herpes simplex in oral ulcers in neutropenic patients. Br J Cancer **61:**469-70.
79. **Khodarev, N. N., S. J. Advani, N. Gupta, B. Roizman, and R. R. Weichselbaum.** 1999. Accumulation of specific RNAs encoding transcriptional factors and stress response proteins against a background of severe depletion of cellular RNAs in cells infected with herpes simplex virus 1. Proc Natl Acad Sci U S A **96:**12062-7.
80. **Kitamura, H., K. Kanehira, K. Okita, M. Morimatsu, and M. Saito.** 2000. MAIL, a novel nuclear I kappa B protein that potentiates LPS-induced IL-6 production. FEBS Lett **485:**53-6.
81. **Kumar, A., J. Haque, J. Lacoste, J. Hiscott, and B. R. Williams.** 1994. Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. Proc Natl Acad Sci U S A **91:**6288-92.
82. **Lachance, G., S. Levasseur, and P. H. Naccache.** 2002. Chemotactic factor-induced recruitment and activation of Tec family kinases in human neutrophils. Implication of phosphatidynositol 3-kinases. J Biol Chem **277:**21537-41.
83. **Lebon, P.** 1985. Inhibition of herpes simplex virus type 1-induced interferon synthesis by monoclonal antibodies against viral glycoprotein D and by lysosomotropic drugs. J Gen Virol **66 (Pt 12):**2781-6.
84. **Lecellier, C. H., P. Dunoyer, K. Arar, J. Lehmann-Che, S. Eyquem, C. Himber, A. Saib, and O. Voinnet.** 2005. A cellular microRNA mediates antiviral defense in human cells. Science **308:**557-60.
85. **Lees, D. N., A. Baskerville, L. M. Cropper, and D. W. Brown.** 1991. Herpesvirus simiae (B virus) antibody response and virus shedding in experimental primary infection of cynomolgus monkeys. Lab Anim Sci **41:**360-4.

86. **Lekstrom-Himes, J. A., J. I. Gallin.** 2000. Molecular basis of phagocyte immunodeficiencies. N. Engl. J. Med **343:**1703.
87. **Li, H., J. Zhang, A. Kumar, M. Zheng, S. S. Atherton, and F. S. Yu.** 2006. Herpes simplex virus 1 infection induces the expression of proinflammatory cytokines, interferons and TLR7 in human corneal epithelial cells. Immunology **117:**167-76.
88. **Li, S., and J. M. Sedivy.** 1993. Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex. Proc Natl Acad Sci U S A **90:**9247-51.
89. **Liu, K. D., S. L. Gaffen, and M. A. Goldsmith.** 1998. JAK/STAT signaling by cytokine receptors. Curr Opin Immunol **10:**271-8.
90. **Liu, Y., S. K. Shaw, S. Ma, L. Yang, F. W. Luscinskas, and C. A. Parkos.** 2004. Regulation of leukocyte transmigration: cell surface interactions and signaling events. J Immunol **172:**7-13.
91. **Love, F. M., and E. Jungherr.** 1962. Occupational infection with virus B of monkeys. Jama **179:**804-6.
92. **Ludwig, H., Pauli, G., Gelderblom, H. et al.** 1983. B virus (Herpesvirus simiae). In: Roizman B. ed. The Herpesviruses. Vol 2 New Yourk: Plenum Press.**:**385-428.
93. **Marsters, S. A., T. M. Ayres, M. Skubatch, C. L. Gray, M. Rothe, and A. Ashkenazi.** 1997. Herpesvirus entry mediator, a member of the tumor necrosis factor receptor (TNFR) family, interacts with members of the TNFR-associated factor family and activates the transcription factors NF-kappaB and AP-1. J Biol Chem **272:**14029-32.
94. **May, M. J., and S. Ghosh.** 1997. Rel/NF-kappa B and I kappa B proteins: an overview. Semin Cancer Biol **8:**63-73.
95. **McClain, D. S., and A. O. Fuller.** 1994. Cell-specific kinetics and efficiency of herpes simplex virus type 1 entry are determined by two distinct phases of attachment. Virology **198:**690-702.
96. **McClure, H. M., B. Olberding, and L. M. Strozier.** 1973. Disseminated herpesvirus infection in a rhesus monkey (Macaca mulatta). J Med Primatol **2:**190-4.
97. **McLean, T. I., and S. L. Bachenheimer.** 1999. Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. J Virol **73:**8415-26.
98. **Medici, M. A., M. T. Sciortino, D. Perri, C. Amici, E. Avitabile, M. Ciotti, E. Balestrieri, E. De Smaele, G. Franzoso, and A. Mastino.** 2003. Protection by herpes simplex virus glycoprotein D against Fas-mediated apoptosis: role of nuclear factor kappaB. J Biol Chem **278:**36059-67.
99. **Melchjorsen, J., and S. R. Paludan.** 2003. Induction of RANTES/CCL5 by herpes simplex virus is regulated by nuclear factor kappa B and interferon regulatory factor 3. J Gen Virol **84:**2491-5.
100. **Melchjorsen, J., F. S. Pedersen, S. C. Mogensen, and S. R. Paludan.** 2002. Herpes simplex virus selectively induces expression of the CC chemokine

RANTES/CCL5 in macrophages through a mechanism dependent on PKR and ICP0. J Virol **76:**2780-8.

101. **Melnick, J. L., and D. D. Banker.** 1954. Isolation of B virus (herpes group) from the central nervous system of a rhesus monkey. J Exp Med **100:**181-94.
102. **Meyers, R. L., and T. H. Pettit.** 1974. Chemotaxis of polymorphonuclear leukocytes in corneal inflammation: tissue injury in herpes simplex virus infection. Invest Ophthalmol **13:**187-97.
103. **Mikloska, Z., V. A. Danis, S. Adams, A. R. Lloyd, D. L. Adrian, and A. L. Cunningham.** 1998. In vivo production of cytokines and beta (C-C) chemokines in human recurrent herpes simplex lesions--do herpes simplex virus-infected keratinocytes contribute to their production? J Infect Dis **177:**827-38.
104. **Miller, R. A., A. Galecki, and R. J. Shmookler-Reis.** 2001. Interpretation, design, and analysis of gene array expression experiments. J Gerontol A Biol Sci Med Sci **56:**B52-7.
105. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi.** 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell **54:**903-13.
106. **Miyazaki, D., Y. Inoue, K. Araki-Sasaki, Y. Shimomura, Y. Tano, and K. Hayashi.** 1998. Neutrophil chemotaxis induced by corneal epithelial cells after herpes simplex virus type 1 infection. Curr Eye Res **17:**687-93.
107. **Mocsai, A., M. Zhou, F. Meng, V. L. Tybulewicz, and C. A. Lowell.** 2002. Syk is required for integrin signaling in neutrophils. Immunity **16:**547-58.
108. **Mogensen, T. H., and S. R. Paludan.** 2001. Molecular pathways in virus-induced cytokine production. Microbiol Mol Biol Rev **65:**131-50.
109. **Molitor, J. A., W. H. Walker, S. Doerre, D. W. Ballard, and W. C. Greene.** 1990. NF-kappa B: a family of inducible and differentially expressed enhancer-binding proteins in human T cells. Proc Natl Acad Sci U S A **87:**10028-32.
110. **Morrison, L. A.** 2004. The Toll of herpes simplex virus infection. Trends Microbiol **12:**353-6.
111. **Mossman, K. L., P. F. Macgregor, J. J. Rozmus, A. B. Goryachev, A. M. Edwards, and J. R. Smiley.** 2001. Herpes simplex virus triggers and then disarms a host antiviral response. J Virol **75:**750-8.
112. **Motoyama, M., S. Yamazaki, A. Eto-Kimura, K. Takeshige, and T. Muta.** 2005. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. J Biol Chem **280:**7444-51.
113. **Nagler, F. P., and M. Klotz.** 1958. A fatal B virus infection in a person subject to recurrent herpes labialis. Can Med Assoc J **79:**743-5.
114. **Nolan, G. P., and D. Baltimore.** 1992. The inhibitory ankyrin and activator Rel proteins. Curr Opin Genet Dev **2:**211-20.
115. **Nolan, G. P., T. Fujita, K. Bhatia, C. Huppi, H. C. Liou, M. L. Scott, and D. Baltimore.** 1993. The bcl-3 proto-oncogene encodes a nuclear I kappa B-like

molecule that preferentially interacts with NF-kappa B p50 and p52 in a phosphorylation-dependent manner. Mol Cell Biol **13:**3557-66.

116. **Oberman, F., and A. Panet.** 1988. Inhibition of transcription of herpes simplex virus immediate early genes in interferon-treated human cells. J Gen Virol **69 (Pt 6):**1167-77.
117. **Orcutt, R. P., G. J. Pucak, H. L. Foster, J. T. Kilcourse, and T. Ferrell.** 1976. Multiple testing for the detection of B virus antibody in specially handled rhesus monkeys after capture from virgin trapping grounds. Lab Anim Sci **26:**70-4.
118. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene **18:**6853-66.
119. **Palmer, A. E.** 1987. B virus, Herpesvirus simiae: historical perspective. J Med Primatol **16:**99-130.
120. **Paludan, S. R.** 2001. Requirements for the induction of interleukin-6 by herpes simplex virus-infected leukocytes. J Virol **75:**8008-15.
121. **Paludan, S. R., S. Ellermann-Eriksen, V. Kruys, and S. C. Mogensen.** 2001. Expression of TNF-alpha by herpes simplex virus-infected macrophages is regulated by a dual mechanism: transcriptional regulation by NF-kappa B and activating transcription factor 2/Jun and translational regulation through the AU-rich region of the 3' untranslated region. J Immunol **167:**2202-8.
122. **Patel, A., J. Hanson, T. I. McLean, J. Olgiate, M. Hilton, W. E. Miller, and S. L. Bachenheimer.** 1998. Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. Virology **247:**212-22.
123. **Perelygina, L., L. Zhu, H. Zurkuhlen, R. Mills, M. Borodovsky, and J. K. Hilliard.** 2003. Complete sequence and comparative analysis of the genome of herpes B virus (Cercopithecine herpesvirus 1) from a rhesus monkey. J Virol **77:**6167-77.
124. **Pfeffer, S., A. Sewer, M. Lagos-Quintana, R. Sheridan, C. Sander, F. A. Grasser, L. F. van Dyk, C. K. Ho, S. Shuman, M. Chien, J. J. Russo, J. Ju, G. Randall, B. D. Lindenbach, C. M. Rice, V. Simon, D. D. Ho, M. Zavolan, and T. Tuschl.** 2005. Identification of microRNAs of the herpesvirus family. Nat Methods **2:**269-76.
125. **Pfeffer, S., M. Zavolan, F. A. Grasser, M. Chien, J. J. Russo, J. Ju, B. John, A. J. Enright, D. Marks, C. Sander, and T. Tuschl.** 2004. Identification of virus-encoded microRNAs. Science **304:**734-6.
126. **Posavad, C. M., and K. L. Rosenthal.** 1992. Herpes simplex virus-infected human fibroblasts are resistant to and inhibit cytotoxic T-lymphocyte activity. J Virol **66:**6264-72.
127. **Prevention, C. f. D. C. a.** 1998. Fatal Cercopithecine herpesvirus 1 (B virus) infection following a mucocutaneous exposure and interim rcommendations for worker protection. MMWR Morb Mortal Wkly Rep **47:**1073-6, 1083.
128. **Ptasznik, A., A. Traynor-Kaplan, and G. M. Bokoch.** 1995. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase.Shc adapter protein signaling complexes. J Biol Chem **270:**19969-73.

129. **Ray, N., and L. W. Enquist.** 2004. Transcriptional response of a common permissive cell type to infection by two diverse alphaherpesviruses. J Virol **78:**3489-501.
130. **Rocha, S., A. M. Martin, D. W. Meek, and N. D. Perkins.** 2003. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-kappaB subunit with histone deacetylase 1. Mol Cell Biol **23:**4713-27.
131. **Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf.** 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology **16:**201-17.
132. **Rosler, A., M. Pohl, H. J. Braune, W. H. Oertel, D. Gemsa, and H. Sprenger.** 1998. Time course of chemokines in the cerebrospinal fluid and serum during herpes simplex type 1 encephalitis. J Neurol Sci **157:**82-9.
133. **Ruebner, B. H., D. Kevereux, M. Rorvik, C. Espana, and J. F. Brown.** 1975. Ultrastructure of Herpesvirus simiae (Herpes B ivurs). Exp Mol Pathol **22:**317-25.
134. **Ryseck, R. P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B. Mol Cell Biol **12:**674-84.
135. **Sabin, A.** 1934. Studies on the B virus. I. The immunological identity of a virus isolated from a human case of ascending myelitis associated with visceral necrosis. . Br J Exp Pathol **15:**248-268.
136. **Sabin, A.** 1934. Studies on the B virus. II. Properties of the virus and pathogenesis of the experimental disease in rabbits. Br J Exp Pathol **15:**269-279.
137. **Sabin, A.** 1934. Sutdies on the B virus. III. The experimental disease in macacus rhesus monkeys. Br J Exp Pathol **15:**321-334.
138. **Sabin, A. B.** 1949. Fatal B virus encephalomyelitis in a physician working with monkeys. J Clin Invest **28:**808.
139. **Sabin, A. B., Wright, A.M.** 1934. Acute ascending myelitis following a monkey bite, with the isolation of a virus capable of reproducing the disease. J Exp Med **59:**115-136.
140. **Samols, M. A., J. Hu, R. L. Skalsky, and R. Renne.** 2005. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. J Virol **79:**9301-5.
141. **Scheidereit, C.** 2006. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene **25:**6685-705.
142. **Scott, M. L., T. Fujita, H. C. Liou, G. P. Nolan, and D. Baltimore.** 1993. The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. Genes Dev **7:**1266-76.
143. **Senftleben, U., Y. Cao, G. Xiao, F. R. Greten, G. Krahn, G. Bonizzi, Y. Chen, Y. Hu, A. Fong, S. C. Sun, and M. Karin.** 2001. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science **293:**1495-9.

144. **Servant, G., O. D. Weiner, P. Herzmark, T. Balla, J. W. Sedat, and H. R. Bourne.** 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science **287:**1037-40.
145. **Shah, K. V., and J. A. Morrison.** 1969. Comparison of three rhesus groups for antibody patterns to some viruses: absence of active simian virus 40 transmission in the free-ranging rhesus of Cayo Santiago. Am J Epidemiol **89:**308-15.
146. **Shawar, S. M., R. R. Rich, and E. L. Becker.** 1995. Peptides from the amino-terminus of mouse mitochondrially encoded NADH dehydrogenase subunit 1 are potent chemoattractants. Biochem Biophys Res Commun **211:**812-8.
147. **Siebenlist, U., G. Franzoso, and K. Brown.** 1994. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol **10:**405-55.
148. **Silverman, N., and T. Maniatis.** 2001. NF-kappaB signaling pathways in mammalian and insect innate immunity. Genes Dev **15:**2321-42.
149. **Stones, P. B.** 1968. Some diseases of animals communicable to man in Britain. In: Graham-Jones O, ed. Proceedings of a symposium organized by the British Small Animal Veterinary Association (Lodon). New York: Pergamon Press.**:**200-1.
150. **Sun, S. C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. Science **259:**1912-5.
151. **Taddeo, B., A. Esclatine, and B. Roizman.** 2002. The patterns of accumulation of cellular RNAs in cells infected with a wild-type and a mutant herpes simplex virus 1 lacking the virion host shutoff gene. Proc Natl Acad Sci U S A **99:**17031-6.
152. **Takeda, K., and S. Akira.** 2004. TLR signaling pathways. Semin Immunol **16:**3-9.
153. **Thompson, J. E., R. J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh.** 1995. I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. Cell **80:**573-82.
154. **Torseth, J. W., and T. C. Merigan.** 1986. Significance of local gamma interferon in recurrent herpes simplex infection. J Infect Dis **153:**979-84.
155. **Totzke, G., F. Essmann, S. Pohlmann, C. Lindenblatt, R. U. Janicke, and K. Schulze-Osthoff.** 2006. A novel member of the Ikappa B family, human Ikappa B-zeta , inhibits transactivation of p65 and its DNA binding. J Biol Chem.
156. **Tumpey, T. M., S. H. Chen, J. E. Oakes, and R. N. Lausch.** 1996. Neutrophil-mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. J Virol **70:**898-904.
157. **U.S. Department of Health and Human Services, C. f. D. C. a. P., and National Institutes of Health.** 2006. Biosafety in Microbiological and Biomedical Laboratories. . US Government Printing Office, Washington, 5th ed.
158. **Vilcek, J., and Sen, J.** 2001. Interferons and other cytokines. In "Virology (Fields BN, Knipe DM, and Howley PM Eds.). Raven, Philadelphia. 4ed.

159. **Wang, F., P. Herzmark, O. D. Weiner, S. Srinivasan, G. Servant, and H. R. Bourne.** 2002. Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. Nat Cell Biol **4:**513-8.
160. **Watanabe, D., A. Adachi, Y. Tomita, M. Yamamoto, M. Kobayashi, and Y. Nishiyama.** 1999. The role of polymorphonuclear leukocyte infiltration in herpes simplex virus infection of murine skin. Arch Dermatol Res **291:**28-36.
161. **Weigler, B. J.** 1992. Biology of B virus in macaque and human hosts: a review. Clin Infect Dis **14:**555-67.
162. **Weigler, B. J., J. A. Roberts, D. W. Hird, N. W. Lerche, and J. K. Hilliard.** 1990. A cross sectional survey for B virus antibody in a colony of group housed rhesus macaques. Lab Anim Sci **40:**257-61.
163. **Welch, H., C. Mauran, and I. I. Maridonneau-Parini.** 1996. Nonreceptor Protein-Tyrosine Kinases in Neutrophil Activation. Methods **9:**607-18.
164. **Westerheide, S. D., M. W. Mayo, V. Anest, J. L. Hanson, and A. S. Baldwin, Jr.** 2001. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. Mol Cell Biol **21:**8428-36.
165. **Whiteside, S. T., J. C. Epinat, N. R. Rice, and A. Israel.** 1997. I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity. Embo J **16:**1413-26.
166. **Whitley, R. J., Hilliard, J.K.** 2001. Cercopithecine Herpesvirus (B virus). In "Virology (Fields BN, Knipe DM, and Howley PM Eds.). Raven, Philadelphia. 4ed.
167. **Wieland, T., and C. K. Chen.** 1999. Regulators of G-protein signalling: a novel protein family involved in timely deactivation and desensitization of signalling via heterotrimeric G proteins. Naunyn Schmiedebergs Arch Pharmacol **360:**14-26.
168. **Wu, L., and P. S. Morahan.** 1992. Macrophages and other nonspecific defenses: role in modulating resistance against herpes simplex virus. Curr Top Microbiol Immunol **179:**89-110.
169. **Yamamoto, M., S. Yamazaki, S. Uematsu, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Kuwata, O. Takeuchi, K. Takeshige, T. Saitoh, S. Yamaoka, N. Yamamoto, S. Yamamoto, T. Muta, K. Takeda, and S. Akira.** 2004. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. Nature **430:**218-22.
170. **Yamazaki, S., T. Muta, S. Matsuo, and K. Takeshige.** 2005. Stimulus-specific induction of a novel nuclear factor-kappaB regulator, IkappaB-zeta, via Toll/Interleukin-1 receptor is mediated by mRNA stabilization. J Biol Chem **280:**1678-87.
171. **Yamazaki, S., T. Muta, and K. Takeshige.** 2001. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. J Biol Chem **276:**27657-62.
172. **Yokota, S., N. Yokosawa, T. Okabayashi, T. Suzutani, S. Miura, K. Jimbow, and N. Fujii.** 2004. Induction of suppressor of cytokine signaling-3 by herpes

simplex virus type 1 contributes to inhibition of the interferon signaling pathway. J Virol **78:**6282-6.

173. **Zachariae, C. O.** 1993. Chemotactic cytokines and inflammation. Biological properties of the lymphocyte and monocyte chemotactic factors ELCF, MCAF and IL-8. Acta Derm Venereol Suppl (Stockh) **181:**1-37.

174. **Zachos, G., B. Clements, and J. Conner.** 1999. Herpes simplex virus type 1 infection stimulates p38/c-Jun N-terminal mitogen-activated protein kinase pathways and activates transcription factor AP-1. J Biol Chem **274:**5097-103.