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B Virus Infection Activates p38 And JNK Pathways Differentially In Cells From Macaque Versus Human Hosts: Exploring Inflammation & Apoptosis

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by

RACHAEL FARAH-ABRAHAM

Under the Direction of Julia K. Hilliard, PhD.

ABSTRACT

B virus (*Macacine herpesvirus* 1), subfamily *Alphaherpesvirinae*, causes a fatal, neuro-virulent infection in zoonotically infected humans. Macaques (*Macaca* sp.) serve as the natural host for B virus and they are frequently seropositive for B virus antibodies without showing any overt signs of disease. The global hypothesis of these studies is that B virus, a highly cytopathic virus in macaques, subverts the innate immune responses in the host (macaques) that has co-evolved with it (the virus) differently than it does the foreign host (humans). The foreign host,

frequently fails to produce neutralizing antibodies early after infection and this may be due to a dysregulation or inhibition of pathways known to play a role in the innate immune response which directs the adaptive defense responses. Current knowledge is that at least five major signaling pathways can be activated after a pathogen such as B virus enters a host cell (REF). These include the IRF3 pathway, the NFkB pathway, the NFAT pathway, and the MAPK pathway. Early stimulation of one or more of these pathways leads to the induction of the proinflammatory response and subsequent induction of cytokines such as IL6, IL8 and IL10, and apoptosis. Cytokine induction and apoptosis play important roles in host-pathogen interactions, innate defense induction and subsequent adaptive immune responses. Using a primary cell model that is representative of the first target cells of B virus in the natural and foreign host, we investigated one of the key signaling pathways, the MAPK pathway, induced by B virus early after infection (Farah-Abraham and Hilliard, unpublished data). My data suggest that macaque and human cells differ in the induction kinetics of MAPK (JNK and p38) activation. These data reveal differences between foreign and natural host cells in how each controls apoptosis, and demonstrate that inhibition of p38 activation reduced and with high dose inhibition terminated B virus replication in human cells, and played a role in reduction of apoptosis-associated mediators. The importance of each component in the MAPK pathway is investigated with respect to virus replication in macaque and human cells that represent the primary target cells in acute infection. Knowledge of these events provides an understanding of how the innate immune responses can be modulated by B virus to shape the adaptive immune response to limit how the virus replicates and spreads. Further, these data may provide insight into a novel target for the design of new antivirals to inhibit this deadly zoonotic virus. This research will help us under-

stand how the early molecular mechanisms of host-pathogen interactions result in modulation of the innate immune responses and how certain aspects of a normally defensive (protective) host response can be re-directed or modified depending on the nature of the virus:host relationship.

INDEX WORDS: B virus, Macaque, Mitogen-activated protein kinase, Apoptosis

B VIRUS SUBVERTS THE INNATE IMMUNE RESPONSE FOR EFFICIENT VIRUS REPLICATION

by

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DEDICATION

There are many individuals to whom this dedication is dedicated, that have stood by me and encouraged me along the way. Your support and encouragement were invaluable in times of pressure and cyclic turmoils, and know that I will always be there to offer your the same support and encouragement in life.

To my mom whose rigor and emphasis on the importance of an education has been a driving force from young, thank you. To my dad who has always been the supportive caregiver and my outlet for a good joke even when I don't feel to smile, thank you.

My hubby, Milli, you know it all best. You are husband and a bestfriend in one. We don't always see eye to eye, but your approach to life and your determination has been a support structure for me, and you have always stood by me in times of need. Thank you for the sacrifices you have made to ensure this goal of mine was completed. More importantly, thank you for bringing Oakley into our life. He offers an outlet for stress relief like no other.

Uncle Joe, if it were not for your constant words of encouragement and generous ways, I may not have even been in pursuit of this degree. You have always been supportive in many ways and treated me as if I were one of your daughters. For this I thank you.

Jonece. Does professional student ring any bells? We have known each other for as long as we have known ourselves and even with the change of location, our friendship was never broken. We can pick up right where we left in a heartbeat and mentored each other in life. As I have found success in my academics I wish you the same in your pursuits in life. Thank you for truly being my best friend, and the sister I never had.

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1 INTRODUCTION (General Introduction)

1.1 Literature Review

Members of the family of viruses classified as *Herpesviridae* can be further divided into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae*. Members of subfamily *Alphaherpesvirinae*, commonly called alphaherpesviruses, are DNA viruses that can be characterized by a short reproductive cycle, efficient destruction of the host cells, the ability to replicate in a wide variety of host tissues and the ability to establish a lifelong latent infection in the sensory ganglia of the peripheral nervous system (PNS) of the natural host (Fields, Knipe et al. 1996; Sandri-Goldin 2006; Weller 2011).

1.2 Virus Characteristics

B virus (*Cercopithecine herpesvirus 1, Macacine herpesvirus 1,* McHV1) is an *alpha-herpesvirus*, native to Old World monkeys in the genus *Macaca*. It has a 156k base pair double-stranded linear DNA genome that is contained within a 160-180 nm icosahedral capsid. This capsid is encapsulated firstly by a matrix of protein called the tegument, followed by an outer lipid envelope that contains many previously described viral glycoproteins, some of which are important for attachment and entry into permissive cells (Ruebner, Kevereux et al. 1975; Ludwig 1983; Perelygina, Zhu et al. 2003; Davison, Eberle et al. 2009; Tregle, Loe et al. 2010). The sequenced B virus lab strain E2490 used in this dissertation work has a genome size of 156789 bp (Perelygina, Zhu et al. 2003). There are 74 identified B virus genes that have 26.6-87.7% amino acid homology to herpes simplex virus type 1 (HSV-1) and Herpes simplex virus type 2 (HSV-2) proteins (Perelygina, Zhu et al. 2003; Zao 2007).

In cell culture, B virus has replication kinetics that is similar to the replication kinetics of HSV-1 and HSV-2 (Whitley 2001). As described for HSV-1 and HSV-2, adsorption and entry into permissive cells occurs during the first hour post infection (p.i.), followed by shut off of host macromolecular synthesis by a tegument protein referred to as virion host shutoff (vhs) (Strelow and Leib 1995). The shut-off of host macromolecular synthesis is followed by a coordinated expression of three classes of viral genes referred to as immediate-early (α), early (β) and late (γ) genes (Honess and Roizman 1974; Roizman, Kozak et al. 1975). Viral DNA replication occurs by 4 hrs p.i. and infectious virus is detectable within 6-10 hours p.i. (Hilliard, Eberle et al. 1987; Whitley 2001).

1.3 Route of Transmission and Pathogenesis

As with other members of the *Alphaherpesvirinae* subfamily, primary B virus infection occurs in mucosal, epithelial, or dermal cells, has a relatively short reproductive cycle followed by retrograde transport of virus within the peripheral nervous system (PNS) from the site of infection, and is capable of establishing latency in the sensory ganglia of infected hosts (Roizman, Carmichael et al. 1981; Aderem and Ulevitch 2000; Akira, Takeda et al. 2001; Farah 2005). Certain stressful stimuli may lead to reactivation of virus, defined by replication of viral DNA and assembly of new particles, followed by anterograde transport of virus particles from the sensory ganglia to epithelial sites (Strelow and Leib 1995; Whitley 2001; Scientific Committee on Health and Environmental Risks 2009). Shedding and transmission of infectious virus can occur intermittently throughout the lifetime of the host.

1.4 Natural Host and Scope of the Problem

Old World monkeys of the genus *Macaca* are the natural hosts of B virus, each having co-evolved together over 30 million years. This genus comprises at least a dozen species and includes some of the earliest recorded non-human primate species used in scientific research due to the macaques genetic, anatomic and physiologic similarities to humans (Valerio, Ellis et al. 1969; Valerio, Pallotta et al. 1969; Farah 2005). Each year 12,000-15,000 macaques are imported into the USA, and they make up 63% of the non-human primates used in research (Capitanio 1998; Council 2002; Conlee KM 2004; J. R. Held 2005; (SCHER) 2009; Scientific Committee on Health and Environmental Risks 2009; Suran and Wolinsky 2009). Macaques have been used in many types of research, including product design, safety testing and infectious agent research. Many major medical discoveries and research contributions in the areas of life cycle, pathogenesis, drug efficacy and vaccine development have been based on research using macaques (Landsteiner 1940; Boen 1964; Goodwin 1970; Fridman 1972; Areekul 1979; Hill 1980; Palmer 1987; Howson 1996; Farah 2005; (SCHER) 2009). Macaques represent an invaluable non-human primate resource in the biomedical community for elucidating disease processes in humans because the macaque immune system offers researchers an opportunity to investigate responses to natural and foreign pathogens at cellular and molecular levels. The recent publication of the rhesus macaque genome sequence will encourage an increased use of macaques for biomedical research (Gibbs, Rogers et al. 2007).

The similarity of pathogens that infect macaques to those that infect other closely related primates such as humans will continue to encourage the use of macaques as research models and the use of the pathogens that infect them as tools in research.

In the wild 73-100% of macaques are known to be seropositive for B virus. The virus can cause a similar disease manifestation in both humans (foreign host) and macaques (natural host), but infections in macaques tend to be benign, whereas infections in humans often lead to an acute infection of the central nervous system (CNS), which may result in fatal encephalitis or serious neurological sequelae (Ostrowski, Leslie et al. 1998; Huff and Barry 2003; Farah 2005). Although symptomatic B virus infection in humans is uncommon, the availability of antiviral drugs such as acyclovir, famciclovir and ganciclovir have allowed progression of the disease in some zoonotically infected patients to be kept under control and/or some neurological symptoms alleviated (Zuniga ; CDC 1987; CDC 1989; Holmes, Hilliard et al. 1990; Ostrowski, Leslie et al. 1998; Tregle, Loe et al. 2010).

1.5 Host Antiviral Response to B Virus Infection

As previously mentioned, B virus infection in macaques (natural host) is similar to that of HSV-1 infection in humans (natural host). In most cases, when a virus such as B virus enters its natural host, the host immune responses are able to control the infection with or without sporadic reactivation throughout the lifetime of the host. Simplex viruses in their natural hosts are cytopathic and the host and virus have selected for properties that will allow the two to coexist together, i.e. the virus replicates periodically and the host continues to support this replication. However, when a virus such as B virus enters a foreign host, the host innate and adaptive immune responses are unable to control the virus in a manner that prevents its entry into the CNS. This phenomenon can also be seen with viruses such as Ebola or Marburg (Barreiro and Quintana-Murci 2010). In these cases the human becomes a terminal host for the virus. Regarding the adaptive immune response to B virus infection, in macaques an IgM response can

be detected within three to ten days p.i., followed by an IgG response around ten to fourteen days. However, this response is induced to a relatively lower level than normally observed and delayed in zoonotically infected humans, if detected at all. For some B virus-infected humans, detectable levels of B virus-specific IgG antibodies can be intermittently observed, suggesting periodic reactivation of B virus, as shown by work from the National B Virus Resource Center (Hilliard 2005). Neutralizing antibodies associated with the infection also appear in both humans and macaques, although at lower levels in humans (Lees, Baskerville et al. 1991; Whitley 2001).

Studies in our laboratory have shown that B virus counteracts the innate immune response by inhibiting protein kinase RNA-activated (PKR) activity, inhibiting NF-κB regulated immune responsive genes putatively by IκBζ and subverting natural killer (NK) cell activity (Zao 2007; Zhu 2007; Vasireddi 2009) (Figure 1). Collectively, the information gained provides a framework within which one can not only understand the pathogenesis of B virus in both the foreign and natural hosts, but, more importantly, can also examine how closely related viruses that have co-evolved with their natural hosts modulate the innate and adaptive immune responses such that both host and virus survive.

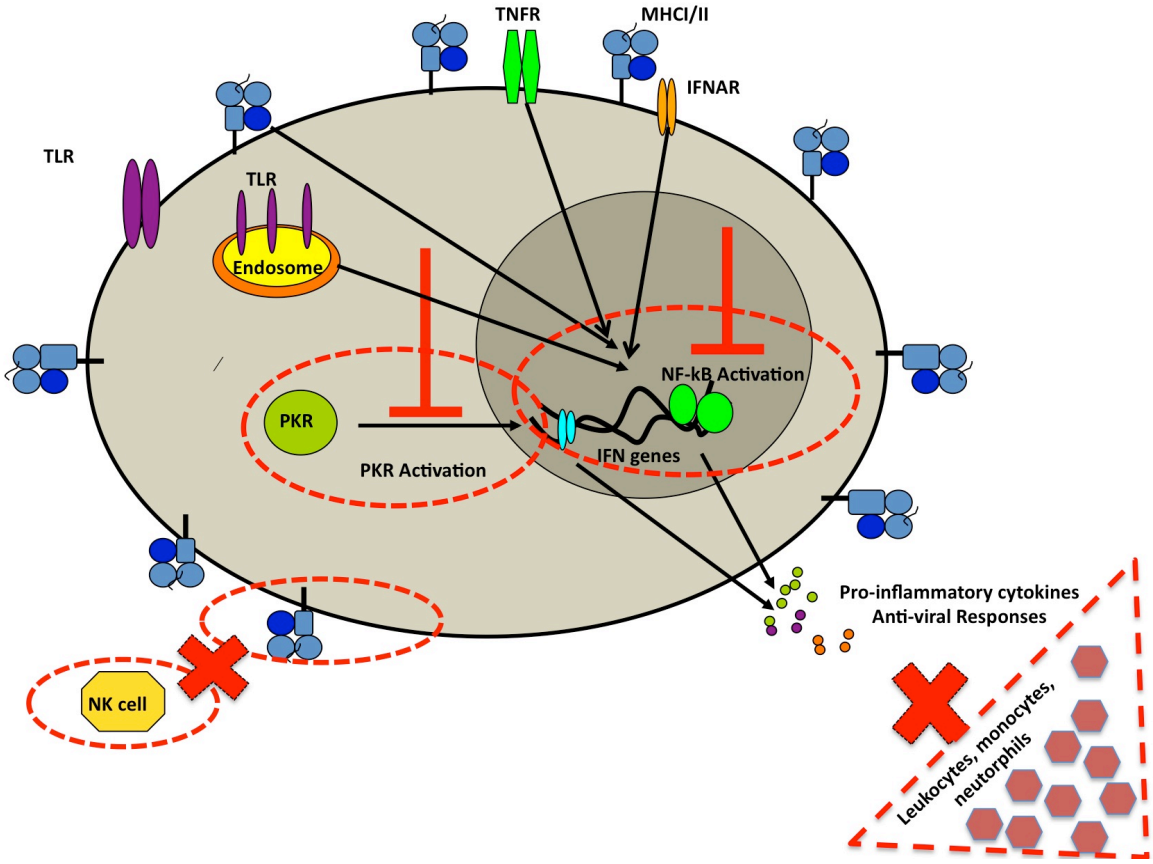


Figure 1. Mechanisms by which B virus counteracts the innate immune response: A summary of investigations to date. (Zao 2007; Zhu 2007; Vasireddi 2009)

Cellular signaling pathways known to be upregulated early after infection can be activated at any and/or every step during virus entry and replication. However, it is still not known how all of these inter-related signaling pathways and their interaction with B virus effect B virus replication and direct the observed adaptive immune responses characteristic of each host species. The fact that B virus did not co-evolve with humans, coupled with evidence gathered from the studies of other viruses, suggests that *neither the virus nor the host has had the opportunity to select for traits that allow the host and virus to co-exist successfully*, i.e., an effective adaptive immune response as seen in the natural host, capable of restricting virus replication and entry into the CNS

In any cell type, the response to virus infection is equal to the sum of cellular signaling events involved in the innate immune response and their downstream outcome, i.e. the secretion of inflammatory cytokines that direct the outcome of the adaptive responses. This downstream inflammatory response then influences the adaptive response and thus determines the overall fate of the host. In order to understand the immune modulation mechanisms of B virus in cells from both macaques and humans, it is important to investigate the mechanisms by which B virus directs a strong humoral defense in macaques, whereas in most humans, it rapidly gains entry into the CNS, causing ascending paralysis and death while suppressing any apparent adaptive defenses.

1.6 Strategies Used by Alphaherpesviruses to Subvert the Innate Immune Response

As previously mentioned, HSV-1 is a closely related alphaherpesvirus, and, as with B virus in the natural host macaques, HSV-1 is able to replicate and persist throughout the lifetime

of its natural host, humans. It has been shown by *in vitro* studies that virally encoded proteins of HSV-1 have antiviral functions. Among these proteins are the following: virion host shut off (vhs), infected cell protein 0 (ICP0), infected cell protein 27 (ICP27), infected cell protein 34.5 (ICP34.5) and US11 (Vandevenne, Sadzot-Delvaux et al. 2010).

The HSV-1 vhs protein is present on the tegument of the virus and has endoribonuclease activity which functions to inhibit the host cell's translation machinery by degrading mRNA (Kwong and Frenkel 1987; Strom and Frenkel 1987; Kwong, Kruper et al. 1988; Whitley 2001; Vandevenne, Sadzot-Delvaux et al. 2010). Whether B virus vhs plays a similar role in infected cells has not been determined at present.

Among the functions of HSV-1 ICP0 protein there is a E3 ubiquitin-like ligase activity which functions to inhibit the host cell's interferon response by disrupting ND10 structures and sequestering transcription factors such as IRF3, and thus blocking interferon beta (IFNβ) gene transcription (Gelman and Silverstein 1986; Whitley 2001; Vandevenne, Sadzot-Delvaux et al. 2010).. Several aspects of ICP0 may influence the role of MAPK in B virus infection: the ability to ubiquitinate proteins targeted for presentation by the MHC I; ubiquitinate the cytoplasmic domain of receptors important in the innate immune response to infection; and modification of cellular proteins via ubiqutination for the activation or degradation of proteins, can all influence the role of MAPK in B virus infection.

Another HSV-1 protein that has antiviral function is ICP27 which inhibits the host cell's transcriptional machinery by inhibiting RNA splicing and it thus prevents the expression of interferon stimulating genes as well as inhibits STAT1 phosphorylation and nuclear translocation

which are important for interferon gene expression (Gelman and Silverstein 1985; Sacks, Greene et al. 1985; Rice and Knipe 1988; Sekulovich, Leary et al. 1988; Estridge, Kemp et al. 1989; Bolovan, Sawtell et al. 1994; Whitley 2001; Vandevenne, Sadzot-Delvaux et al. 2010). The alteration of the kinetics production and/or activation of proteins involved in the cytokine gene expression influenced by MAPK could putatively increase cell survival times long enough for virus replication and spread to neighboring cells and establishment of latency in neurons.

Similarly, HSV-1 ICP34.5 protein prevents: type I interferon expression through inhibition of IRF3 activation and host cell antiviral PKR responses to infection. This is accomplished by dephosphorylation of cellular protein, eukaryotic translation initiation factor 2-alpha kinase 2 (eIF2α), thus driving host cell protein synthesis (Gelman and Silverstein 1985; Bolovan, Sawtell et al. 1994; Whitley 2001; Vandevenne, Sadzot-Delvaux et al. 2010). The MAPK pathway has many functional targets that can play a role in the promotion of viral gene expression and contribute to the overall efficiency of viral replication in host cells, thus a potential role of ICP27 and other immediate early proteins could be to stabilize cellular transcripts necessary for cell survival and enhance viral replication. B virus, however, lacks this protein and appears to replace at least part of its function via the encoded US11 protein.

HSV-1 US11 protein is a tegument protein, which also inhibits the PKR response by blocking its activation. Additionally it inhibits dsRNA activation of latent RNase, RNase L, by blocking oligoadenylate synthase activity (OAS) (Roller and Roizman 1990; Roller and Roizman 1992; Cassady, Gross et al. 1998; Whitley 2001; Vandevenne, Sadzot-Delvaux et al. 2010). In HSV-1 US11 is expressed late in infection; however the B virus homolog of this protein is ex-

pressed as an early gene product. Early expression of US11 can be observed in ICP34.5 deletion mutants of HSV-1 (Cassady, Gross et al. 1998).

In summary, simplexviruses encode a number of proteins that enable effective virus replication by delaying cell death. However, these same proteins via their expression kinetics serve to ultimately limit virus replication perhaps for the benefit of host survival, enabling a relatively peaceful coexistence of these viruses within their natural hosts.

1.7 Cell Signaling Events that Occur In Response to a Pathogen

When host physical barriers, e.g., skin, mucous, secretory antibodies, to infection have been circumvented, cells then rely on the sensing of pathogens through a group of transmembrane proteins including Toll-like receptors (TLRs) (Aderem and Ulevitch 2000; Akira, Takeda et al. 2001). These membrane receptors have a heterogeneous group of ligands that can be cell-type dependent and function on or within a cell to stimulate similar signaling cascades through their interaction with cytoplasmic adaptor molecules and other scaffold proteins involved in the innate immune response (Kaisho and Akira 2001). These receptors once activated, especially TLRs play an important role in orchestrating the molecular signals through induction of a universal and/or specific response to the invading pathogen (Barreiro and Quintana-Murci 2010). After molecular signals linked to the innate immune response have been initiated, there are many cytoplasmic mediators that play key roles in the integration and progression of the response for effective clearance of the pathogen and development of an antigen-specific adaptive response.

Of the many signaling pathways that exist within a cell, the stimulation of four well-known intracellular cascades can occur after infection to modulate cell death (apoptosis) and trigger the release of cytokines (Figure 2). They are the nuclear factor kappa-B (NF-κB) cascade, the mitogen-activated protein kinase (MAPK) cascade, the interferon regulatory factor (IRF) pathway and the nuclear factor of activated T-cells (NF-AT) (Takeda, Kaisho et al. 2003).

Some of these pathways rely on a feedback mechanism or biphasic response mechanisms to amplify the signaling, and collectively they allow for transcription factors such as IRF3, NF-κB and AP1 to be upregulated for gene transcription (Vandevenne, Sadzot-Delvaux et al. 2010). Since it is known that a common strategy of viruses to promote viral infection is to manipulate, and or exploit preexisting intra-cellular pathways, it is therefore important to investigate how certain aspects of a normally protective host responses are being re-directed, modified or inhibited to the advantage of a host that has co-evolved with a specific virus – in this case, macaques and B virus – and to its disadvantage in a foreign host, specifically humans infected with B virus.

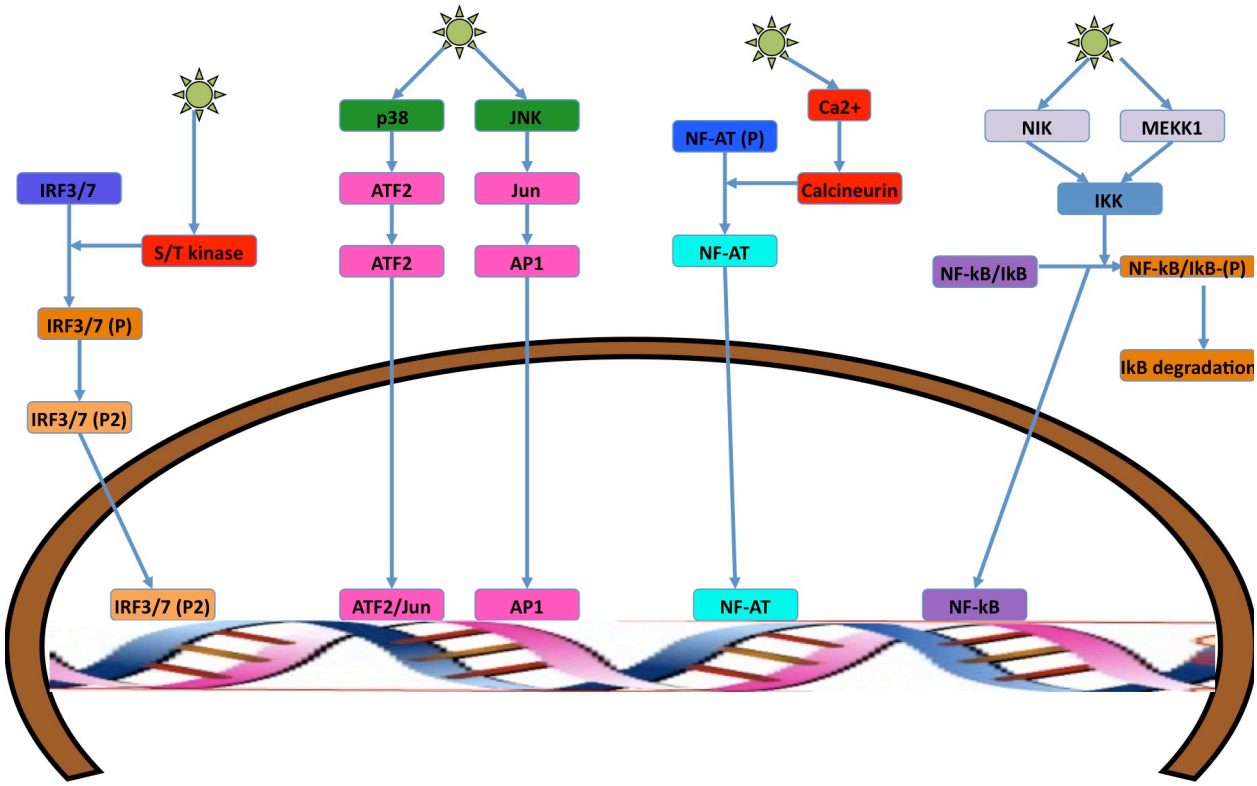


Figure 2 A summary of the currently understood main early signaling pathways associated with host cell detection of virus infection.

1.8 Protein Complexes that Mediate Activation of Signaling Pathways

As previously mentioned, stimulation of PRRs can activate four intracellular protein kinase cascades resulting in the induction of type I IFN's, apoptosis and many inflammatory cytokines that collectively play a role in the innate immune response (Takeda, Kaisho et al. 2003). TLR signals are mediated by a conserved Toll–interleukin 1 (IL-1) receptor domain (TIR) in the cytoplasmic regions that recruits adaptor molecules such as myeloid differentiation factor (MyD88), TIR-associated protein (TIRAP) (also called MAL), Toll receptor-associated activator of

interferon (TRIF) and Toll receptor-associated molecule (TRAM) (Su 2005). These adaptor molecules function to recruit and assemble additional intracellular signaling molecules, including IL-1 receptor-associated kinases (IRAK) and tumor necrosis receptor factor 6 (TRAF6), into complexes (Su 2005). Toll-like receptor-associated activator of interferon (TRIF), a multipurpose adaptor protein that acts as a platform for the recruitment of several proteins (IKK, TBK1, VISA and TRAF6), has been shown to be involved in the activation of the interferon (IFN) response and the MAPK-induced apoptotic response (Meylan, Burns et al. 2004; Zhong, Tien et al. 2006) (Figure 3). TRIF can also interact with an adaptor protein called Fas-associated death domain protein (FADD), which activates caspase 8 leading to the subsequent activation of caspase 3 and induction of apoptosis (Kreuz, Siegmund et al. 2004; Yoneyama, Kikuchi et al. 2004; Kato, Takeuchi et al. 2006; Zhong, Tien et al. 2006) (Figure 3).

Another functionally important adaptor protein, TIRAP/MAL, has been shown to interact with the TLR-mediated MyD88-dependent pathway for inflammatory cytokine production as well as NF-κB and JNK activation (Takeda and Akira 2004). Within this pathway, the TIRAP-MyD88 complex activates TRAF6 through the IL1 receptor kinases (IRAK's). Activated TRAF6 is linked to TAK1 (MAP3K7) through adaptor protein TAK1 binding protein (TAB2) leading to stimulation of the JNK pathway and cellular activator protein-1 (AP1) activation (Silverman and Fitzgerald 2004; Kishida, Sanjo et al. 2005) (Figure 3). In the studies described in this dissertation, an initial goal was to identify the adaptor molecules upstream of the MAPK pathways that were critical to the activation of MAPK following B virus infection of macaque and human cells and to verify whether cells from each species responded similarly following B virus infection.

Several members of the MAP3K family, including TAK1 (MAP3K7), tumor progression locus-2 (Tpl2) and ASK1, have been shown to be essential in mediating TLR-activated kinase cascades. Because the activation of a MAPK is downstream of a MAP2K and MAP3K, it is possible that one or more MAP3Ks are required for activation of the MAPK cascades after TLR stimulation (Su 2005). Two such MAP3Ks, called ASK1 (apoptosis-inducing signaling kinase 1) and TAK1, are capable of activating both the p38 and JNK pathways (Ichijo, Nishida et al. 1997; Kishida, Sanjo et al. 2005) (Figure 3) and these were investigated in this dissertation.

Other proteins that function independently of the TLR pathways, but are able to act as dsRNA detectors within the cytoplasm, are the retinoic acid inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 product (MDA5/helicard). Both RIG-I and MDA5 contain two caspase-recruitment domains (CARDs), which recruit a CARD-containing adaptor protein called VISA (also known as MAVS, IPS-1 or Cardif) (Kato, Takeuchi et al. 2006; Takeuchi and Akira 2008). VISA relays the signal to kinases TBK1, IKK, receptor-interacting protein kinase1 (RIP 1) and TRAF6 for the phosphorylation of downstream transcription factors that are involved in the expression of type-I interferons, proinflammatory cytokines and apoptotic events (Meylan, Tschopp et al. 2006; Yoshida, Takaesu et al. 2008) (Figure 3).

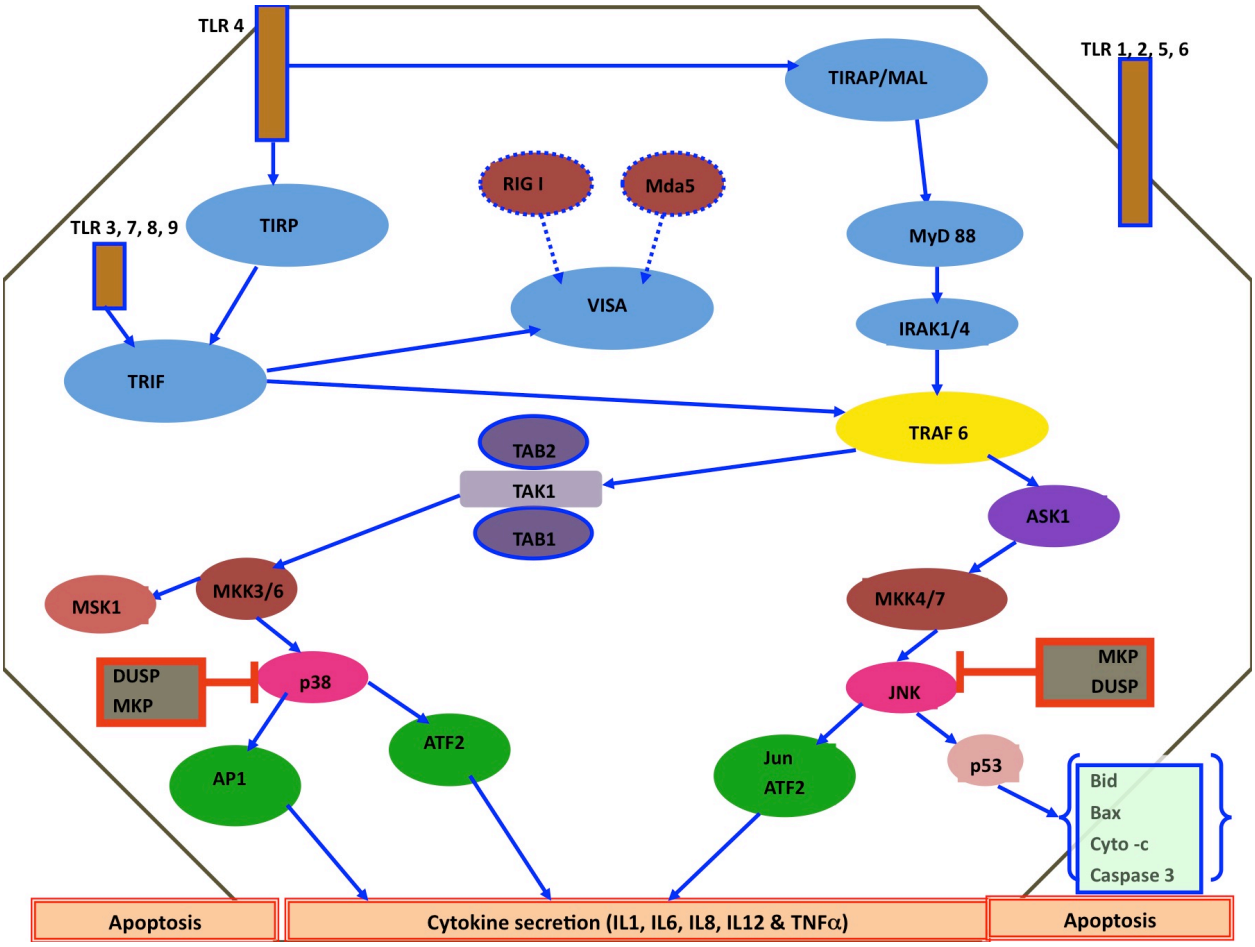


Figure 3 Protein complexes that mediate activation of signaling pathways

1.9 Activation of Intracellular Pathways

To detect an invading pathogen, the innate immune response depends on many different evolutionally conserved recognition mechanisms. Collectively, these recognition mechanisms rely on molecular patterns known as pattern recognition receptors (PRRs) that recognize specific pathogen-associated molecular patterns (PAMPs) that are not present in host cells. Examples of PAMPs are viral DNA, single-stranded ssRNA, dsRNA and glycoproteins of varied pathogens (Kato, Takeuchi et al. 2006). Four main families of PRRs have been shown to initiate

proinflammatory signaling pathways: the Toll-like receptors (TLRs), the NOD-like receptors (NLRs), the RIG-I-like receptors (RLRs) and the DNA sensors.

The TLRs were the first identified and remain the best-characterized receptors among the signaling PRRs. They initiate key inflammatory responses by recognizing a variety of exogenous PAMPs from bacteria, fungi, parasites, and viruses, such as lipopolysaccharide (LPS), flagellin, and CpG DNA, and also assist in directing specific aspects of the adaptive immune defenses.

RIG-I-like-receptors (RLRs) belong to a family of inducible cytoplasmic RNA helicases thought to be essential for host antiviral responses. Two well-known RNA helicases are RIG-I (retinoic-acid-inducible protein 1) and MDA-5 (melanoma-differentiation-associated gene 5). They function to sense the endogenous PAMPs, such as the replication intermediate for RNA viruses which is double-stranded RNA (dsRNA), and are capable of amplifying the innate immune response to infection (Onoguchi, Yoneyama et al. 2011). Laboratory of genetics and physiology 2 (LGP2) represents a third RLR which functions as a negative regulator of RIG-I and MDA-5 (Onoguchi, Yoneyama et al. 2011; Technologies 2011).

Recently, another group of PRRs called NOD-like receptors (NLRs)/CATERPILLERs, have been identified. NLRs have more than 20 members in mammals and, though not clearly understood, their primary role is to recognize cytoplasmic PAMPs as with the TLRs (Takahashi, Ip et al. 2009; Technologies 2011). NLRs can be further sub-divided into two categories: those that signal via NF-κB and MAPK pathways and those (NALPs) that play a role in the assembly of the inflammasome (Inohara and Nunez 2001; Takahashi, Ip et al. 2009).

Other important players that associate with PRRs either to initiate a proinflammatory response to infection or to induce phagocytosis are the inflammasome, peptidoglycan recognition proteins (PGRP), C-type lectin families, also called C-type lectin receptors (CLRs), and Damage Associated Molecular Patterns (DAMPs) (Kerrigan and Brown 2009; Technologies 2011).

Inflammasomes are large caspase 1 activating complexes formed within the cytoplasm after NLR engagement of PAMPs. These function to cleave prototype inflammatory cytokines proIL-1β and pro IL18 into their active forms (Takahashi, Ip et al. 2009). Currently there are four known inflammasomes, NLRP1 (NALP1), NLRP3 (NALP3 or PYPAF1 or cryopyrin), NLRC4 (IPAF) and AIM2, whose components and stimuli differ (Stutz, Golenbock et al. 2009). Little is known about which of these plays a role in simplexvirus infections, including B virus.

PGRP and CLRs, such as mannose receptor, dectin-1, DNGR-1 and mannose binding lectin, recognize PAMPs and act as phagocytic receptors to mediate or regulate the uptake of pathogens via phagocytosis by binding microbial carbohydrate moieties (glycan structures) not present in the host (Zelensky and Gready 2005; Kerrigan and Brown 2009; Takahashi, Ip et al. 2009; Dam and Brewer 2010).

Danger Associate Molecular Patterns (DAMPs) are endogenously upregulated nuclear and cytoslolic signaling molecules that activate TLRs and innate immune cells via a not-yet-clarified mechanism that is distinct from the co-receptors and accessory molecules utilized by PAMPs (Rubartelli and Lotze 2007; Piccinini and Midwood 2010). DAMPs may activate positive feedback loops that allow for increased TLR activation, thus contributing to increased inflammation (Piccinini and Midwood 2010).

1.10 Mitogen-Activated Protein Kinase Pathway

The key focus of the studies reported in this dissertation involve mitogen-activated protein kinase (MAPK) cascade, which is highly conserved and is involved in many cellular functions including cell proliferation, cell differentiation, cell migration, inflammation and apoptosis following cell infection. External stimuli, such as growth factors, inflammatory cytokines, ligands for G-protein coupled receptors (GPCR), as well as cellular and environmental stress and the presence of dsRNA, have been shown to induce the sequential activation of the MAPK phosphorylation cascade. This cascade begins with activation of the most membrane-proximal MAPK enzyme commonly referred to as a MAP kinase kinase kinase (MAP3K) and ends at the terminal mitogen activated protein kinase (MAPK), which phosphorylates many substrates. These substrates include transcription factors such as AP1 heterodimers (activating transcription factor-2/ATF2), Fos, Jun and other protein kinases such as MAPK-activated protein kinases (MAPKAPKs) (Perelygina, Zhu et al. 2003; Ashwell 2006). The overall regulation of downstream events that are influenced by MAPK pathways involves successive phosphorylation and dephosphorylation events of MAPKs. Subsequent nuclear translocation events and modulations of transcription factors can then lead to stimulus-dependent modifications of gene expression to regulate the antiviral response which involves the induction of type I IFN's, cytokines and apoptosis (Chu, Ostertag et al. 1999; Nishimoto and Nishida 2006) (Figure 4).

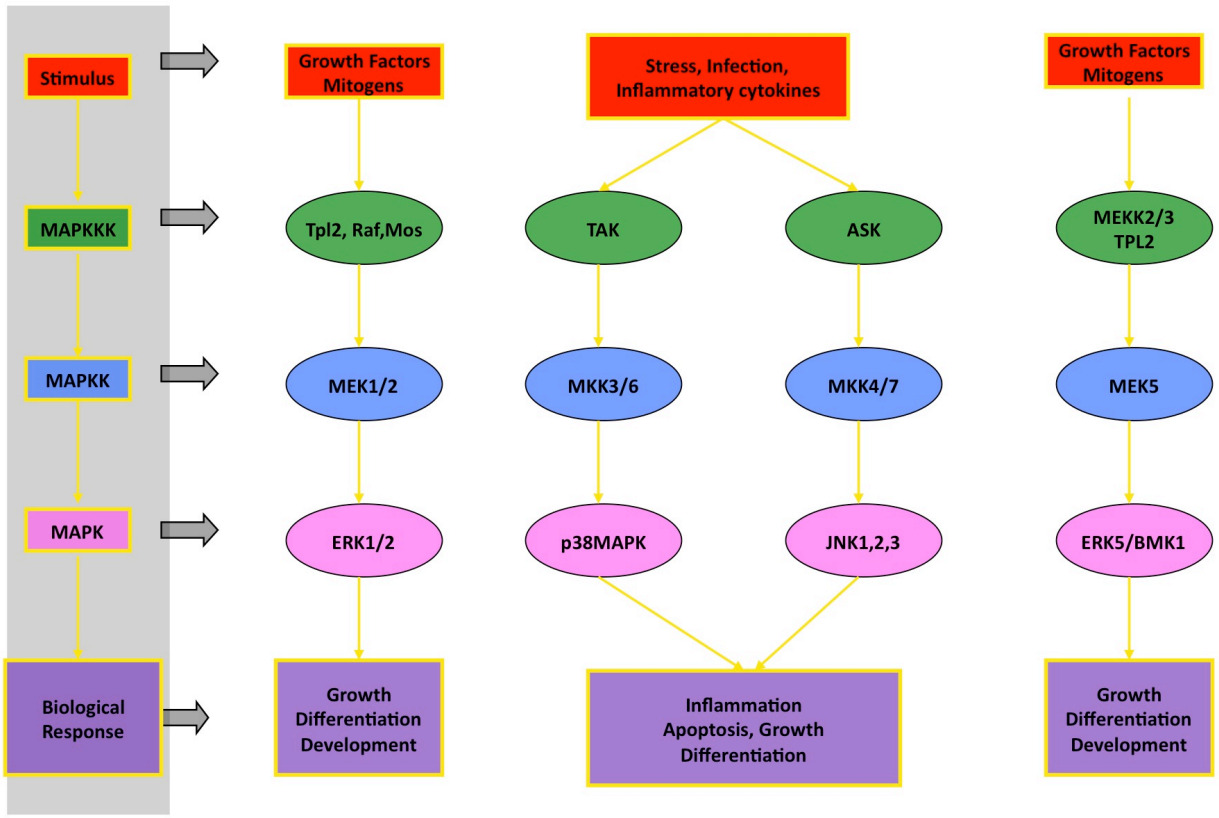


Figure 4. A representation of the Mitogen-Activated Protein Kinase (MAPK) signaling pathway

Currently 14 MAP3K isoforms and seven MAP2K isoforms have been identified. The fourteen MAP3K isoforms are diverse, and can be regulated in a differentially depending on the initial stimuli, whereas the seven MAP2K isoforms are highly specific to their substrates, with little variation in the MAP2K-to-MAPK level of the cascade (Zhang and Dong 2005). The MAPKs may be regulated by MAP kinase phosphatases (MKP) or dual-specificity phosphatases (DUSP) that act as antagonists (negative regulators) to control the magnitude and duration of their activation. To date, 13 MKPs have been identified, each of which having a specific sub-cellular location, substrate specificity and mode of regulation (Zhang and Dong 2005).

The MAPK family consists of four sub-families: extracellular-signal-related kinase 1/2 (ERK1/2), extracellular-signal-related kinase 5 (ERK5), p38 MAPK, and Jun N-terminal kinase (JNK). Of these four MAPK families, the p38 and JNK pathways are commonly referred to as the stress-activated MAP kinase pathways (SAPK) because inflammatory cytokines, environmental stresses, DNA damaging agents and viral infection can induce their activity and these are the targets of the studies presented in the following sections.

The p38 MAPK pathway is activated by MAP3Ks (MTK1, ASK1, TAK1) that exist upstream of p38. These MAP3Ks then phosphorylate and activate the MAP2Ks (MAP2K3 and MAP2K6) (Ashwell 2006). Once activated, p38 acts on transcriptional targets such as c-Jun and c-Fos. There are four isoforms of the p38 MAPK (p38α, p38β, p38γ and p38δ) which may be differentially regulated under certain circumstances and differ in tissue distribution, regulation of kinase activation and subsequent phosphorylation of downstream substrates (Dominguez, Powers et al. 2005). MAPK p38α is the most thoroughly studied MAPK p38 isoform, and both

p38α and p38β respond to many of the same agonists (Tibbles and Woodgett 1999). Hence, the p38 MAPK pathway represents a point of convergence for multiple signaling processes that are activated during any cellular stress, infection by a pathogen and inflammation. The pathway is involved in the induction of apoptotic events and the biosynthesis of tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β), IL-6 and IL-12 at the transcriptional and translational level (Dominguez, Powers et al. 2005) (Figure 3 and Figure 5).

The second SAPK pathway is the JNK pathway. The JNK pathway is evolutionarily conserved and can be activated in many cell types in response to inflammatory cytokines, cellular stress and viral infection (Chu, Ostertag et al. 1999; Zhang and Dong 2005). JNK has three isoforms, JNK1, 2 and 3, where JNK3 is brain-specific (Davis 1994; Hu, Qiu et al. 1997; Mielke, Damm et al. 2000). JNKs can phosphorylate transcriptional targets c-Jun and ATF2, leading to an induction of type I IFN's, production of inflammatory cytokines (IL-1, IL-6, IL-12 and TNFα) and apoptosis (Chu, Ostertag et al. 1999; Zhang and Dong 2005). A number of upstream kinases have been implicated in the activation of JNK: ME2K1; ME2K4; apoptotsis signal-regulating kinase (ASK1); mixed lineage kinases (MLK); and TGFβ activating kinase (TAK1). Scaffolding proteins, such as JNK interacting protein (JIP), β-arrestin and JNK stress-activated protein (JSAP1), have also been identified. The overall function of scaffolding proteins is to tether signaling proteins in the vicinity of each other, allowing the assembly of specific complexes that further direct the subsequent signaling pathways (Lin and Dibling 2002) (Figure 3 and Figure 5).

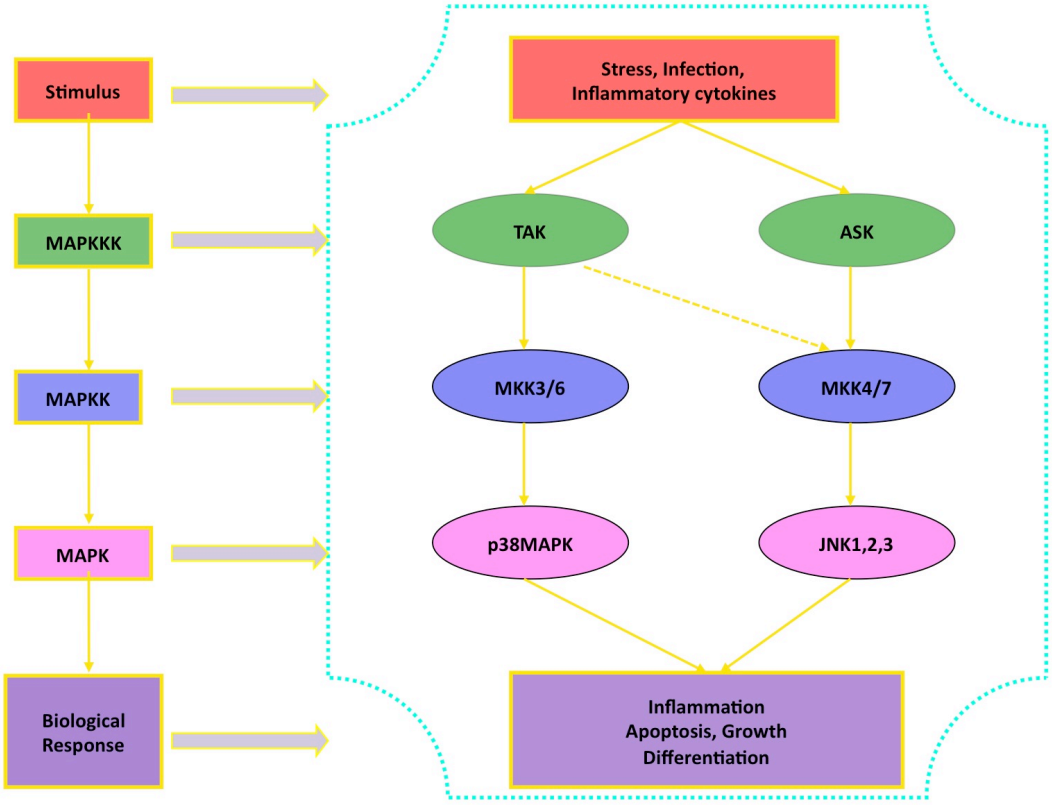


Figure 5. A representation of Stress Activated Protein Kinases (SAPK's) signaling pathway

The activities of both the JNK and p38 pathways can be regulated positively or negatively by a number of phosphatases called MAPK phosphatases (MKPs), or by protein-protein interactions that prevent nuclear translocation events (Tibbles and Woodgett 1999; Lin and Dibling 2002).

The extracellular signal-regulated kinase pathways ERK1/2 and ERK5 are similar in activation modes and functions, as they are stimulated by growth factors and mitogens. These two pathways are essential in cellular growth, differentiation and development. As with the SAPKs,

the ERKs can interact with regulatory elements such as scaffold proteins and MAPK phosphatases that regulate their activity.

1.11 MAPK-Dependent Apoptosis

Apoptotic induction and regulation is important in host-pathogen interactions and in immune responses to infection. Studies have suggested that the evolutionary conserved MAPK-dependent apoptosis plays a critical role in innate immune responses to viral infection in eukaryotic cells (Sumbayev and Yasinska 2006). The MAPK family commonly referred to as SAPKs (p38 and JNK) are known to play a critical role in mediating the decision of cell fate by regulating apoptosis. Apoptosis signal-regulating kinase (ASK1) is a ubiquitously expressed MAP3K that activates the SAPKs p38 and JNK pathways by phosphorylating their upstream MAP2K. In the case of inactivated JNK (non-phosphorylated JNK), JNK is complexed with p53, which is unstable and degraded by the proteosome (Sumbayev and Yasinska 2006). Activated p38 and JNK are able to phosphorylate transcription factors c-Jun and ATF-2. The Jun protein may then form a homodimer (Jun-Jun) or it may form heterodimers with ATF2 to form Jun-ATF2 (AP1) (Sumbayev and Yasinska 2006). Later transcription factors and other nuclear transcription factors can lead to increased expression of the c-Fos protein, which is able to dimerize with c-Jun to further enhance overall AP1 levels (Tibbles and Woodgett 1999). As a result, the apoptotic and innate immune response to viral infection initiated by ASK1 activation of the SAPK pathways can lead to downstream effects that involve caspase 3 activation and apoptosis (Sumbayev and Yasinska 2006).

1.12 Virus Stimulation of MAPK Pathways

As previously mentioned, B virus is an alphaherpesvirus, indigenous to Old World monkeys in the genus *Macaca*. Following B virus entry into host cells, uncoating, transport and viral replication occurs to permit a triphasic (temporal) transcription of the immediate-early (α), early (β), and late (γ) genes. When a pathogen such as B virus infects a cell, it sets off a program of gene expression by conveying a signal along one or more pathways, some of which can be protein kinase pathways, to act on downstream transcription factors. It is known that innate immune response plays a large role in the initial ability of a host to eliminate a pathogen. This initial response leads to the activation of cell-mediated cytotoxic responses that are triggered by varied pattern recognition receptors (membrane-bound or cytoplasmic) and involves the stimulation of multiple pathways for the secretion of type I IFN's, cytokines and the upregulation of transcription factors involved in cell survival, cell proliferation and cell death (apoptosis). Of these pathways, the mitogen-activated protein kinase (MAPK) signaling pathways have been shown to play an essential role in the innate immune response to viral infection (Chu, Ostertag et al. 1999; Young and Dillin 2004; Cooray, Jin et al. 2005; Zhang and Dong 2005; Sumbayev and Yasinska 2006).

MAPKs represent a molecular signaling network present in all cell types that is altered in immunological, inflammatory and degenerative diseases. Studies have shown that all three subfamilies of the *herpesviruses* can activate one or more of the MAPK pathways during infection; however, it is not known which events within signaling pathways regulate the induction of transcription factors that mediate the innate immune response (Xie, Pan et al. 2005; Sloan, Han et al. 2006). With *herpesvirus* infections, the cell-cycle regulatory machinery and other intracellu-

lar signaling pathways of the host cell are altered not only to promote cell-cycle progression but also to facilitate viral replication and spread.

As with any pathogen that enters a host (natural or foreign), induction of host cell signaling pathways and other biochemical changes occur that can be characterized as a molecular signature which is a direct result of the specific invading pathogen. This signature begins at the initiation of infection and varies depending on the pathogen and the host. The ability of the infected cell to sense an invading pathogen and stimulate the immune response or induce cell death is thus of great importance. Without the ability of the cell to recognize invasion, either as a result of pathogen-associated inhibitors or the absence of pathogen recognition receptors results in the ability of the virus to replicate unfettered and untoward consequences for the defenseless host.

In summary, we know that viral infections are detected by pathogen recognition receptors and this detection results in the activation of intracellular signaling pathways, such as protein kinase cascades, that are modulated or inhibited to affect cellular function and to enhance viral replication. We also know that many viruses inclusive of *herpesviruses* generally induce different components of the MAPK pathway. At the onset of this research, no data were available that described what pathways were induced in cells infected with B virus, much less what differences existed in different cell species' responses to B virus infection. Without this knowledge, little headway can be made with respect to development of successful antiviral interventions that minimize morbidity and mortality during zoonotic infections. To begin this research, experiments were designed to test the hypothesis that B virus induces specific innate immune

responses when it enters the initial target cells populating epidermal and dermal layers of skin from humans versus macaques and that these responses affect virus replication levels that ultimately lead to differential induction of cytokines and chemokines that direct the adaptive defenses observed in each host system. Hypothesis: Host-specific innate immune responses triggered by B virus infection are modulated in a host-dependent manner that results in modulation of virus replication.

The mitogen-activated protein kinase (MAPK) pathway was the rational choice for study as the components of the pathway act as multifunctional networks that play a role in cellular responses to cell growth and differentiation, stress, apoptosis and proinflammatory cytokine secretion. Activation of one or more MAPKs has been shown to occur in all three subfamilies of human *herpesviruses*, and the MAPKs can provide an alternate route for the induction of inflammatory cytokines after infection that distinct from that of IRF3 and NFkB induction (Meusel and Imani 2003; Hargett, McLean et al. 2005). Because we know that viruses depend on host cells for their replication and that they tend to reorganize or inhibit cellular functions such as the MAPK pathway to modulate cellular and viral gene expression, I focused these studies on the induction and regulation of the MAPK pathway and its components to identify specific cellular defenses and the effect of these on virus replication.

I tested this hypothesis by investigating the following areas: (1) identification and comparison of B virus signaling in target cells from the natural and foreign host, (2) quantification of the effect of signaling on virus replication and (3) analysis of the outcomes in adaptive host re-

sponses induced as a result of specific signaling events. The following specific aims were designed to test the previously stated hypothesis:

Aim 1: Establish *in vitro* target cell models representing the cells that are initially infected during acute infection in order to study early virus:host interactions.

Aim 2: Identify the specific MAPK responses induced by B virus in acutely infected cells.

Aim 3: Measure MAPK-induced gene products and proinflammatory mediators that are upregulated in response to MAPK-adaptor molecule activation to establish whether these events correlate with observed adaptive responses characteristic of each host species defenses against B virus infection.

2 Aim 1: Establish *in vitro* target cell models representing the cells that are initially infected during acute infection in order to study early virus:host interactions.

2.1 Introduction

Typically with zoonotic (macaque to human) infection with B virus direct animal to human contact occurs through of a bite or scratch. Indirect human exposure can involve situations in which contaminated water or other solutions comes into contact with an open skin wound, or a work-related accidental injury (such as a scratch from the metal door of a cage). In any case where the initial barrier of the skin was broken and an infection occurred, one of the first

cell populations that the virus encounters and can infect is either epidermal cells in the epidermal skin layers and/or fibroblasts in the dermal layers of the skin. Replication may occur in these cell types prior to the retrograde transport of virus by sensory neurons innervating the region. Virus may reach the dendrites and axons without replication, but replication at the initial site of contact ensures a robust population of virus that can come into contact with the peripheral nerves. This initial virus replication also results in activation of innate host defenses which in turn direct adaptive defense responses. Without virus replication there is no opportunity for the induction of immunological defenses, leaving the host defenseless during reactivated infections.

The skin is our largest organ and comprises varied cell types, nerves, glands and layers. Generally it is described as being made up of three layers: epidermis, dermis and subcutis (subcutaneous) (Figure 6). Each layer contains essential cell types characteristic of that layer. Cell types are found in two forms: immature cell types (whose names end in “-blast”) secrete the fibers and ground substances of the matrix; mature and terminally differentiated cell types (whose names end in “-cyte”) function to maintain the matrix (Phillip 2001).

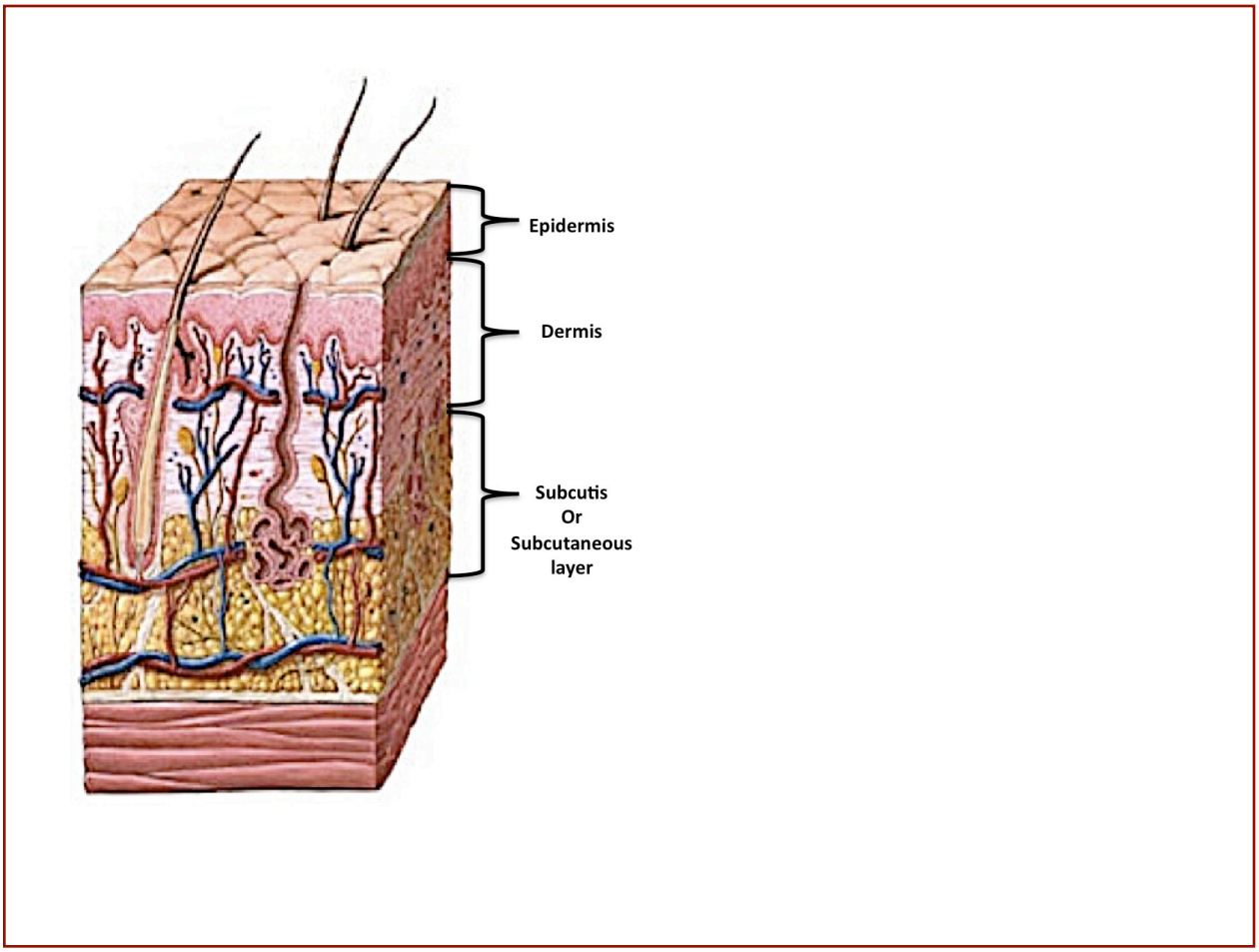


Figure 6 Anatomy of the layers of the skin (a)

The skin is the largest organ and comprises of a complete mixture of cell types, nerves, glands and layers (epidermis, dermis and subcutis/subcutaneous tissue).

Reference: File:HumanSkinDiagram.xcf

It is within the layers of the epidermis and dermis that keratinocytes and fibroblasts reside, respectively (Figure 7). They represent the largely distributed population of cells that comprise the majority cell type of their respective dermal layer and play a role in the skin's innate immune response to any pathogen (McGrath 2004). These cell types play a key role in skin innate immunity by first recognizing pathogens via their membrane-bound and intracellular

pattern recognition receptors and then by secreting cytokines, growth factors, antimicrobial peptides and other inflammatory mediators. The secretion of cytokines is critical to the host cell's inflammatory responses, the recruitment of macrophages and neutrophils, and the development of the adaptive response.

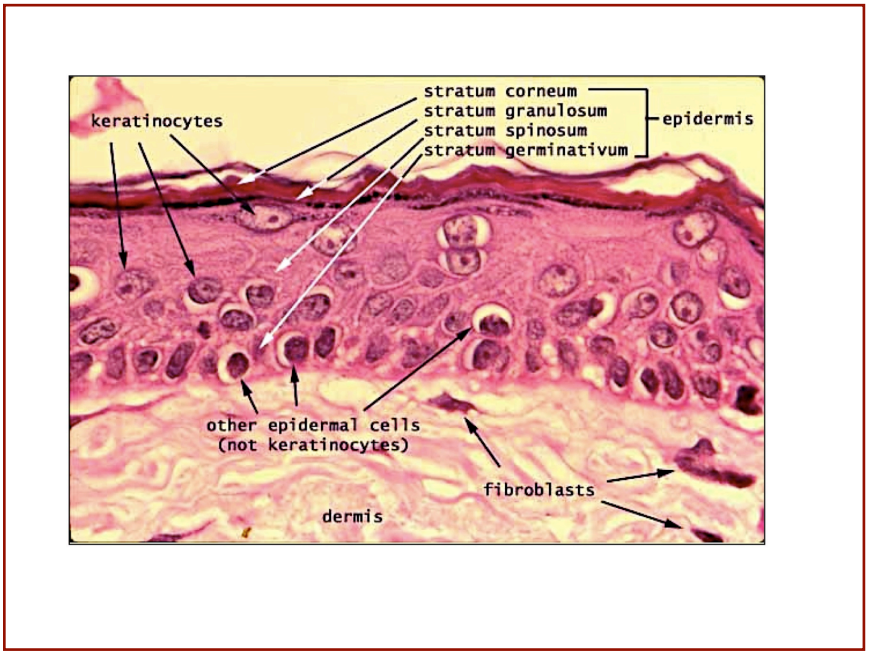


Figure 7 Anatomy of the layers of the skin (b)

It is within the layers of the skin that we find keratinocytes and fibroblasts.

Reference: http://www.siumed.edu/%7Edking2/intro/skin.htm

2.2 Epidermis

The epithelial layer of the skin serves as a protective barrier against pathogens from the outside environment. It is a four-layered stratified, non-vascularized structure. Its thickness ranges from 0.1 mm to 1.4 mm (Weston 2007) (Gawkrodger 2002; Kanitakis 2002; Akgun 2010)

The upper (outermost) layer of cells are continuously renewed from cells of the innermost layer of the epithelium (basal) as the cells divide and are pushed upward to the surface (outermost layer) (Gawkrodger 2002; Kanitakis 2002; Weston William L 2007; Akgun 2010). Differentiated cells, called keratinocytes because they produce keratin, comprise the majority cell type of the epidermis. Preliminary experiments (unpublished) have shown that keratinocytes are susceptible to B virus infection. Keratinocytes are present in the four layers of the epidermis, each layer consisting of cells that are at a different maturation stage (Fritsch 2008). The uppermost layer of the epidermis is continuously replenished; it takes approximately 30 days for the non-keratinized cells to become keratinized and move from the basal membrane to the uppermost layer (Weston William L 2007). Other cell types distributed within the epidermis are Langerhans cells (3-6%), melanocytes (5-10%), Merkel cells (least quantity) and T cells, all of which have their specialized function (Gawkrodger 2002; Kanitakis 2002; Plonka, Passeron et al. 2009; Akgun 2010).

Langerhans cells are a mobile dendritic cell subset of antigen-presenting cells that take up antigen and present it to resident naïve T cells within the epidermis, activating the T cells and the immune response (Kanitakis 2002; Tuchinda and Gaspari 2010).

Melanocytes are specialized cells that synthesize the pigment melanin. These cells use their dendritic processes to transfer the melanin to basal mitotically active keratinocyte cells (Gawkrodger 2002; Weston William L 2007). The melanin, which rests like a cap over the nuclei of keratinocytes, functions to protect cells from UV rays (Gawkrodger 2002; Kanitakis 2002; Weston 2007; Weston William L 2007).

Merkel cells are cells with neuroendocrine and endothelial features that are in close contact with the innervating sensory nerves of the epidermis (Kanitakis 2002). They appear to play a role in sensation, acting as mechanoreceptors (Gawkrodger 2002; Kanitakis 2002).

T cells with various functions can be found circulating in the epidermis. The different T cells types present are T helper (Th) cells, delayed hypersensitivity T helper cells, cytotoxic T (Tc) cells and suppressor T (Ts) cells (Gawkrodger 2002).

2.3 Keratinocytes

As mentioned previously, keratinocytes represent the majority (85%) cell type in the epidermis (Figure 8). Generated in the basal layer of the epidermis, keratinocytes produce keratin as they mitotically divide and move towards the apical surface of the skin (Reue and Glueck 2001; Tchkonia, Tchoukalova et al. 2005). They have been shown to secrete a plethora of growth factors and cytokines, such as fibroblast growth factor (FGF), colony-stimulating factor (CSF), TGF-α, TGF-β, amphiregulin, platelet-derived growth factor (PDGF), IL-1, IL-3, IL-6, IL-8, and TNF-α (Reue and Glueck 2001; Tchkonia, Tchoukalova et al. 2005). They have also been shown to express MHC class II antigens, to express intracellular adhesion molecule (ICAM-1) and to play a role in tissue homeostasis, wound repair and certain cancers (Reue and Glueck 2001; Tchkonia, Tchoukalova et al. 2005). In the verification and characterization of fibroblast primary cell lines, companies have consistently used immunofluorescencent methods with antibodies to cytokeratins. The cytokeratins group consists of approximately 29 different proteins that are characteristic of epithelial cells and are intermediate filaments of epithelial cells in keratinizing tissue (skin). Cytokeratin is a heterodimer of type I (acidic polypeptides) and type II

(basic polypeptides) keratins; at least one member of the acidic and basic family is expressed in all epithelial cells (Hudson 2002). Commercially available fluorescent antibodies, which have cytoplasmic staining, are frequently used to determine if neoplasms and other tumors originated from an epithelial or non-epithelial source (Hudson 2002).

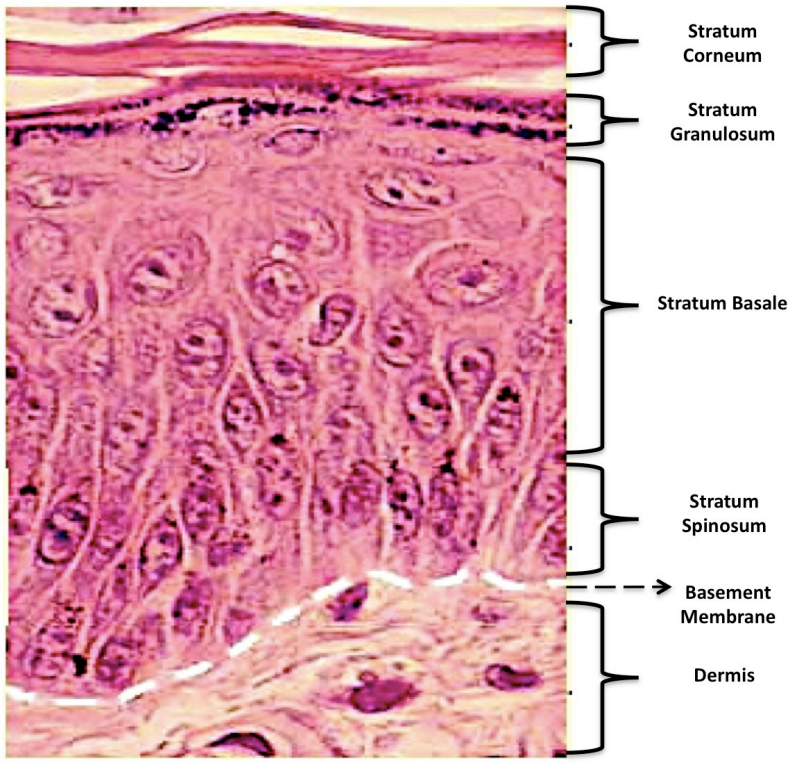


Figure 8. Major cell type within the epidermis

Keratinocytes produce keratin as they mitotically divide and move towards the apical surface of the skin. Shown above are the layers within the epidermis. It is within these layers that the continuous process of keratinization of the cells occurs as they move towards the surface. Thus each layer, basal to upper, represents keratinocytes at a different stage of maturation.

Reference: http://www.siumed.edu/%7Edking2/intro/skin.htm

2.4 Dermis

The dermis is a two-layered supportive connective tissue structure that lies in close contact with the epidermis. Its thickness is between 0.6-3.0 mm and it contains vascularized structures, nerves, nerve endings, hair follicles, sebaceous glands and cells associated with the immune response (Phillip 2001; Weston 2007)(Figure 9). Within the dermis, collagen makes up 70% of the matrix; fibroblasts constitute the majority cell type; dendritic cells are present; and over 90% of the circulating T cells associated with the skin are found (Bos JD and ML. 1987; Gawkrodger 2002; Weston William L 2007). The elasticity within the dermis is provided by a matrix of elastin fibers loosely arranged in many directions, while the collagen (the major structural protein) imparts some rigidity and accounts for 70-80% of the dry weight of the dermis (Gawkrodger 2002). The proteoglycans glycosaminoglycans (GAG), commonly referred to as the 'ground substance' of the dermis, is thought to provide viscosity and hydration to the dermis (Gawkrodger 2002). Chondroitin sulphate represents the major GAG in the dermis followed by dermatan sulphate and hyaluronan (Gawkrodger 2002).

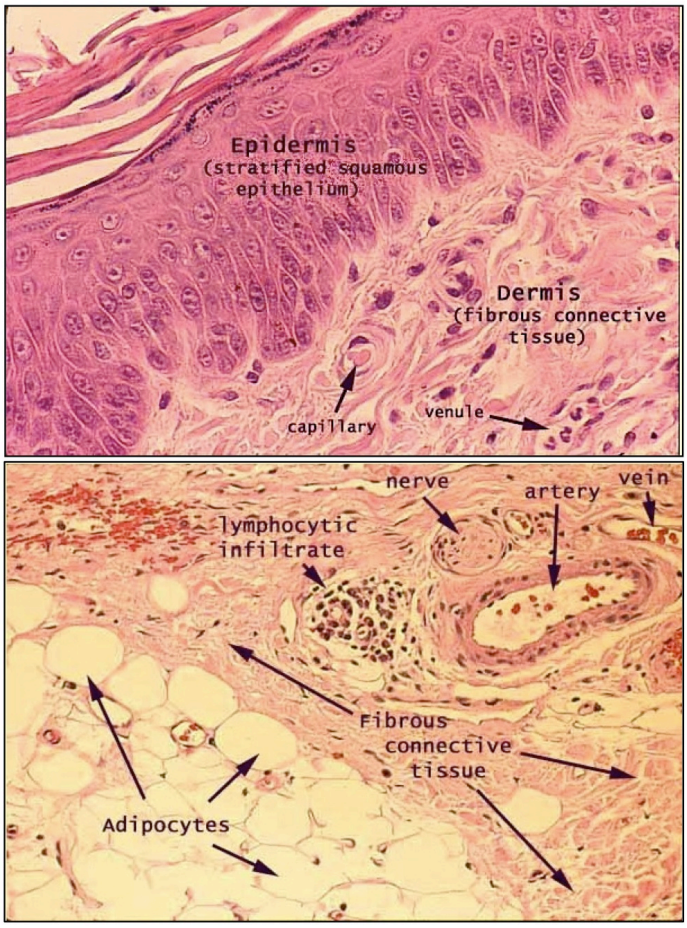


Figure 9 Major cell type within the dermis

Fibroblasts constitute the major cell type of the dermis and are generally the predominant cell type of connective tissues. They secrete collagen, elastin, GAG and other connective tissue elements to create a non-rigid extracellular matrix. Within the dermis one will find other structures such as nerve endings and blood vessels.

Ref: http://www.siumed.edu/%7Edking2/intro/skin.htm

2.5 Fibroblasts

As previously mentioned, fibroblasts constitute the majority cell type in the dermis and are generally the predominant cell type of connective tissues (Bos JD and ML. 1987; Gawkrodger 2002; Kanitakis 2002; Weston William L 2007) (Figure 9). They are mesenchymal-derived cells that can be spindle- (more common) or stellate-shaped and are widely used for in vitro studies of cellular and molecular events in hosts (Gabbiani 1981; Kanitakis 2002; Stair 2004).

Fibroblast primary cell lines for many species are currently available, and they are easy to maintain in culture (Gabbiani 1981; Stair 2004). It has been suggested that fibroblasts in different parts of the body are intrinsically different, and because of these differences care should be taken in deciphering which anatomical location they are derived from when used in a study (Gabbiani 1981; Stair 2004). Fibroblasts secrete collagen, elastin, GAG and other connective tissue elements to create a non-rigid extracellular matrix (Gabbiani 1981; Gawkrodger 2002; Stair 2004).

In the verification and characterization of this type of primary cell line, fibroblast identification and characterization has not only been done by microscopic visualization of their spindle-shaped morphology, but also by immunofluorescencent methods with antibodies to fibronectin, vimentin and/or Te7 antigen. Fibronectin is a glycoprotein present in a soluble di-

meric or multimeric (200 kDa each subunit) form in the matrix and surface of fibroblasts (Gabbiani 1981; Stair 2004).

Langerhans cells are cutaneous immune cells of the epidermis and dermis. They are collectively referred to as cutaneous dendritic cells or professional antigen presenting cells, and are responsible for T-lymphocyte mediated immunity within the skin (Nicolas and Schmitt 1984; Wu, Grouard-Vogel et al. 2000; Valladeau and Saeland 2005). They have the ability to differentiate into mature dendritic cells, present protein-dervived peptide antigens to naive T-lymphocytes, and are able to move between layers of the skin and draining lymph nodes (Valladeau and Saeland 2005) .

It is known that many viruses have co-evolved with their natural host so that the host's immune system and other intra-signaling networks are manipulated to favor virus replication and not cause death. Therefore, it is reasonable to assume that different species would have different responses to infection. To understand the influence virus-host interactions have on host range (natural versus foreign) and the extent of virulence observed within each potential host it is necessary to have cell lines that are representative of similar sites of infection in both the natural and foreign host. These cell lines should be representative of the in vivo situation after infection (at the site of infection), so an accurate analysis and determination of genes that are key regulators of virulence are identified. The use of comparable cell lines from each host (macaque and human) that are from similar locations (epidermal or dermal origin) has two benefits: it enables one to delineate transcriptional responses that occur in the host's (foreign

or natural) response to infection; and it allows one to identify potential points within the cascade of transcriptional responses that can be used as drug targets against infection.

Currently, unlike human fibroblasts, macaque fibroblasts are not available for purchase. Thus, to effectively investigate host responses in primary fibroblast cells derived from the natural (macaque) and foreign (human) host after B virus infection in vitro, it necessitated the establishment of a primary macaque fibroblast cell line. These cells were then used for all subsequent comparative analyses.

3 Specific Aim II: Identify the specific MAPK responses induced by B virus in acutely infected cells.

3.1 Introduction

The initiation of molecular pattern recognition events at the cell surface and in the cytoplasm via the pattern associated molecular patterns (PAMPs) and pathogen recognition receptors (PRRs) occurs when any pathogen enters a host (foreign or natural). The subsequent induction of host cell signaling pathways and other biochemical changes involved in innate immune response can be characterized as a molecular signature. This signature begins at the time of infection and varies depending on the pathogen and the host. The ability of an infected cell to quickly sense the invading pathogen and stimulate the immune response or induce cell death via interconnected downstream signaling pathways is thus of great importance.

The methods employed by pathogens such as viruses to manipulate cellular targets and signaling pathways for their benefit to encourage a productive infection can be considered a

molecular signature of that pathogen. The initial recognition of a virus by a host cell is usually due to genetic and non-genetic viral components that trigger cell signaling events of the innate immune response. Three primary modes have been suggested for triggering the cellular response to infection: the binding of viral glycoproteins to cellular receptors, the presence of *de novo* viral proteins or genomic material, and the expression of viral mRNA or viral genes during their replication cycle (Figure 10) (Greber 2002).

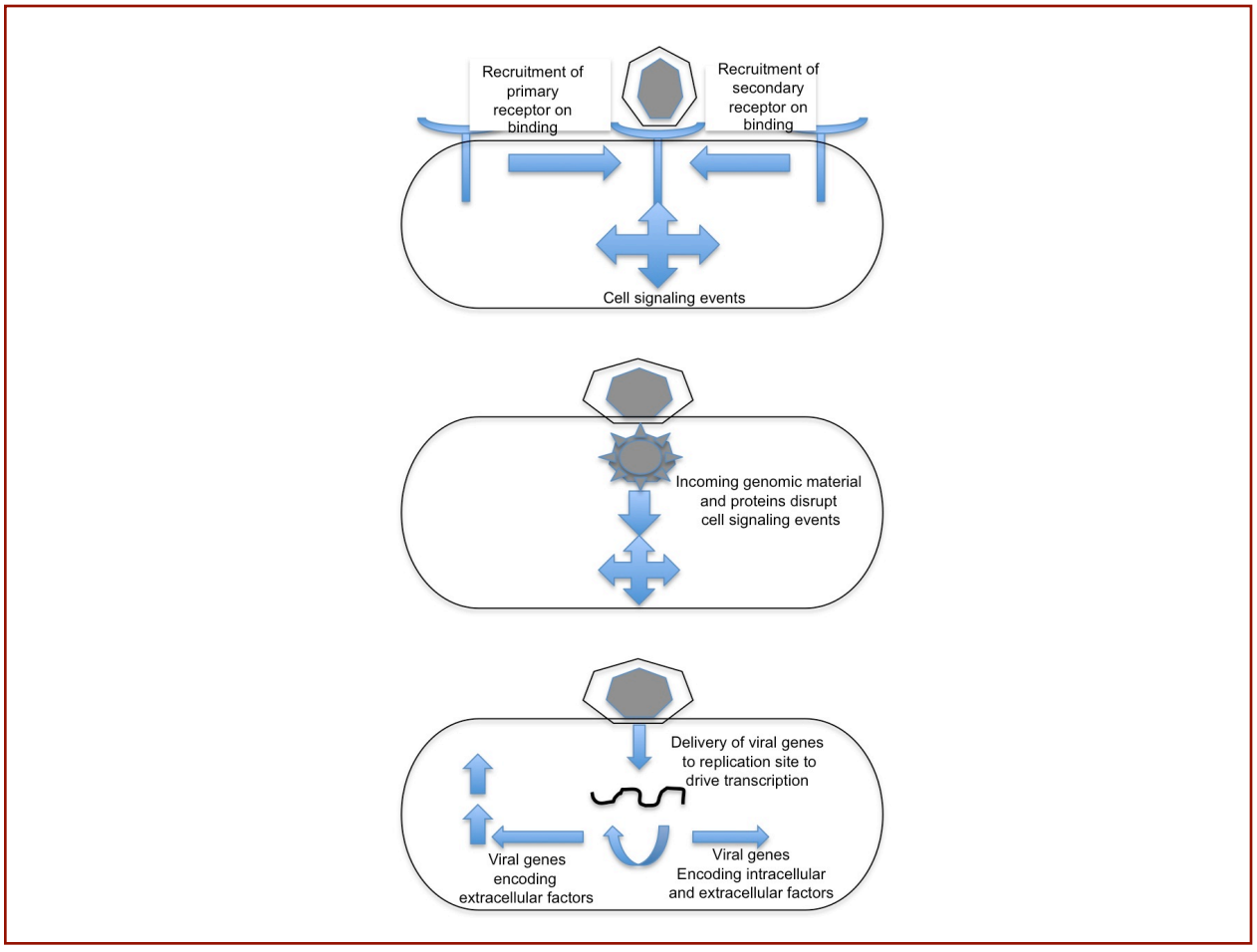


Figure 10 Three modes of viral activation after sensing by pattern recognition receptors

3.2 The first line of defense: detection and activation

Two key events must occur to have an effective innate immune response: first, detection of the pathogen via pattern recognition receptors (PRRs) and, secondly, initiation and activation of protein signaling cascades, leading to cytokine secretion and apoptosis.

Pathogen associated molecular patterns (PAMPs) are evolutionary conserved structures that are present on pathogenic and non-pathogenic organisms (Medzhitov 2007; Rasmussen, Reinert et al. 2009). Within cells they allow for cellular recognition, and discrimination of self (Medzhitov 2007). However, many pathogenic organisms share PAMPs that are recognized by a specified group of nucleotide sensing receptors called pattern recognition receptors (PRRs) (Medzhitov 2007).

PRRs are constitutively expressed in host cells and are able to detect pathogens regardless of their life-cycle stage (Akira, Uematsu et al. 2006). Unlike adaptive immune recognition which is mediated by antigen receptors on varied cell types, PRRs are involved in innate immune recognition and have broad specificity as they function to sense, gather and relay messages to downstream signaling pathways involved in the innate immune response (Medzhitov 2007). The cellular location of the PRR can be at the surface of cell membranes, within the membranes of endosomes and within the cytoplasm of the cell. Toll-like receptors (TLRs) represent the membrane bound PRRs. Examples of cytoplasmic PRRs are TLRs, interferon-inducible dsRNA activated protein kinase/protein kinase R (PKR), 2’5’ Oligoadenylate synthase (2’5’OAS), Nod-like receptors (NLRs), retinoic acid-inducible gene I (RIG I), melanoma differentiation associated gene 5 (MDA5), DNA-dependent activator of IFN-regulatory factors (DAI) and absent in

melanoma 2 (AIM2) (Akira and Takeda 2004; Akira, Uematsu et al. 2006). Each of which has ligand specificity within cells and is not necessarily present in all cells (Akira and Takeda 2004; Akira, Uematsu et al. 2006).

Generally, viruses enter cells by directly binding to receptors on the cell surface and then enter the cytoplasm, via a pH-dependent mechanism involving the endocytotic pathway, or involving endocytosis. Viral glycoproteins, proteins and nucleotide structures are the main PAMPs detected by infected cells. Detection of the viral PAMP by early sensor systems leads to many sequential and non-sequential signaling events for genes involved in the innate and adaptive immune response to be expressed (Akira and Takeda 2004; Akira, Uematsu et al. 2006; Christensen and Thomsen 2009).

3.3 Membrane bound pathogen recognition receptors: Toll-like receptors (TLRs)

Present in plants, invertebrates and vertebrates, Toll-like receptors (TLRs) represent one of the most characterized signaling pathogen recognition receptors (PRRs). TLRs are membrane bound PRRs that are able to detect PAMPs on the cell surface, within the extracellular space and within endosomes (Rasmussen, Reinert et al. 2009). In mammals, there are thirteen members of the TLR family, ten of which are conserved between humans and mice, and it has been suggested that phagocytic cells such as macrophages, neutrophils and dendritic cells constitutively express the highest levels of TLRs (Table 1) (Boehme and Compton 2004; So and Ouchi 2010).

Table 1 Mammalian Toll-like Receptors

TLR	Adapter (s)	Location
TLR 1	MyD88/MAL	Cell surface
TLR 2	MyD88/MAL	Cell surface
TLR 3	TRIF	Cell compartment
TLR 4	MyD88/MAL/TRFI/TRAM	Cell surface
TLR 5	MyD88	Cell surface
TLR 6	MyD88/MAL	Cell surface
TLR 7	MyD88	Cell compartment
TLR 8	MyD88	Cell compartment
TLR 9	MyD88	Cell compartment
TLR 10	Unknown	Cell surface
TLR 11	MyD88	Cell surface
TLR 12	Unknown	unknown
TLR 13	Unknown	unknown

Structurally TLRs are transmembrane proteins with cytoplasmic domains that are homologous with the interleukin (IL)-1 receptor super-family and have leucine rich repeats (LRR) that recognize the PAMP (Medzhitov 2007) (Figures 11 and 12). The homology within their cytoplasmic domain is referred to as the Toll-interleukin receptor (TIR) domain. Ligand binding to TLRs leads to multimerization and receptor-clustering, followed by the assembly of intracellular adaptor molecules each of which has a TIR domain. The combination of the adaptor molecules then allows for specificity in TLR signaling (Figure 13).

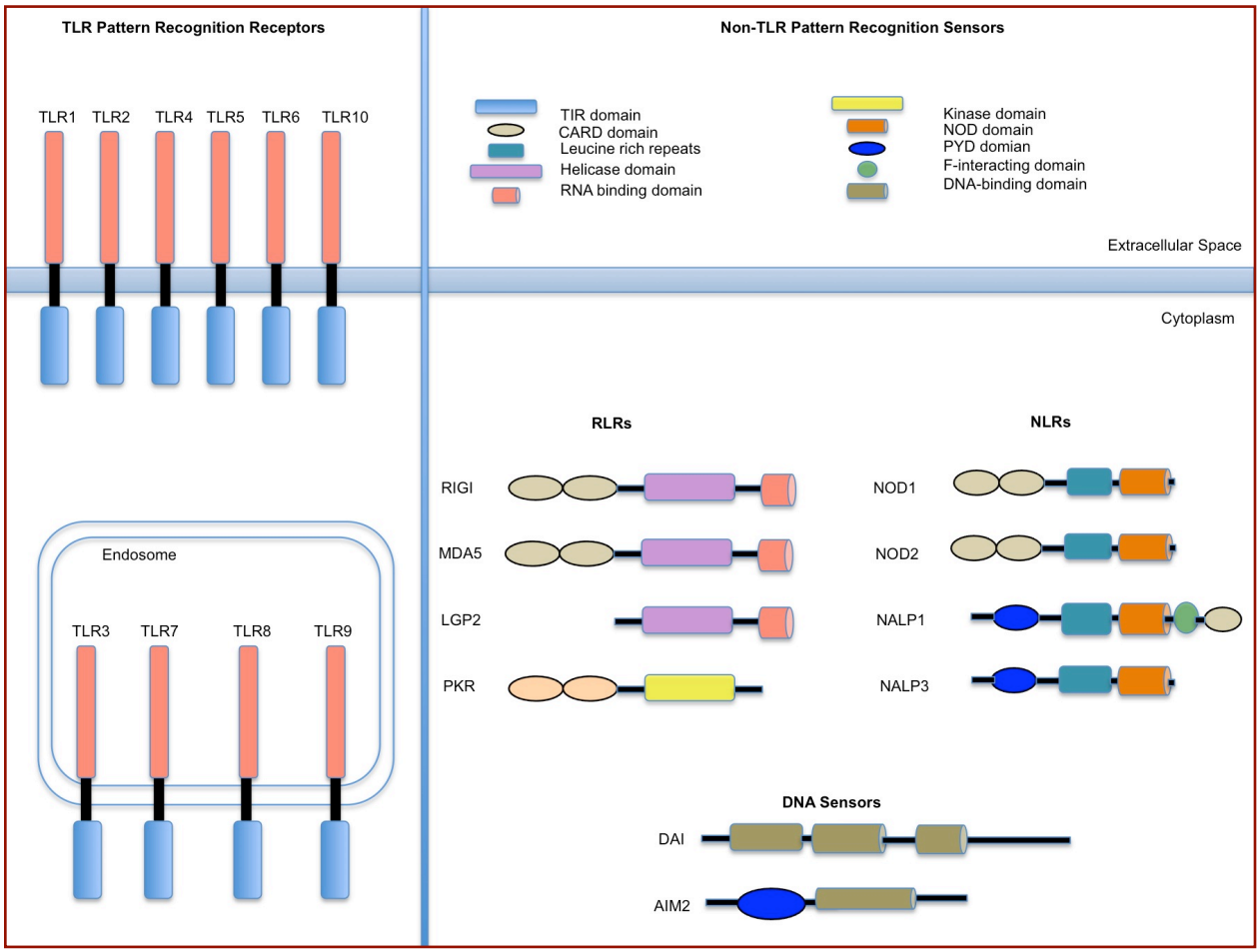


Figure 11 Membrane-bound and non membrane-bound PRR

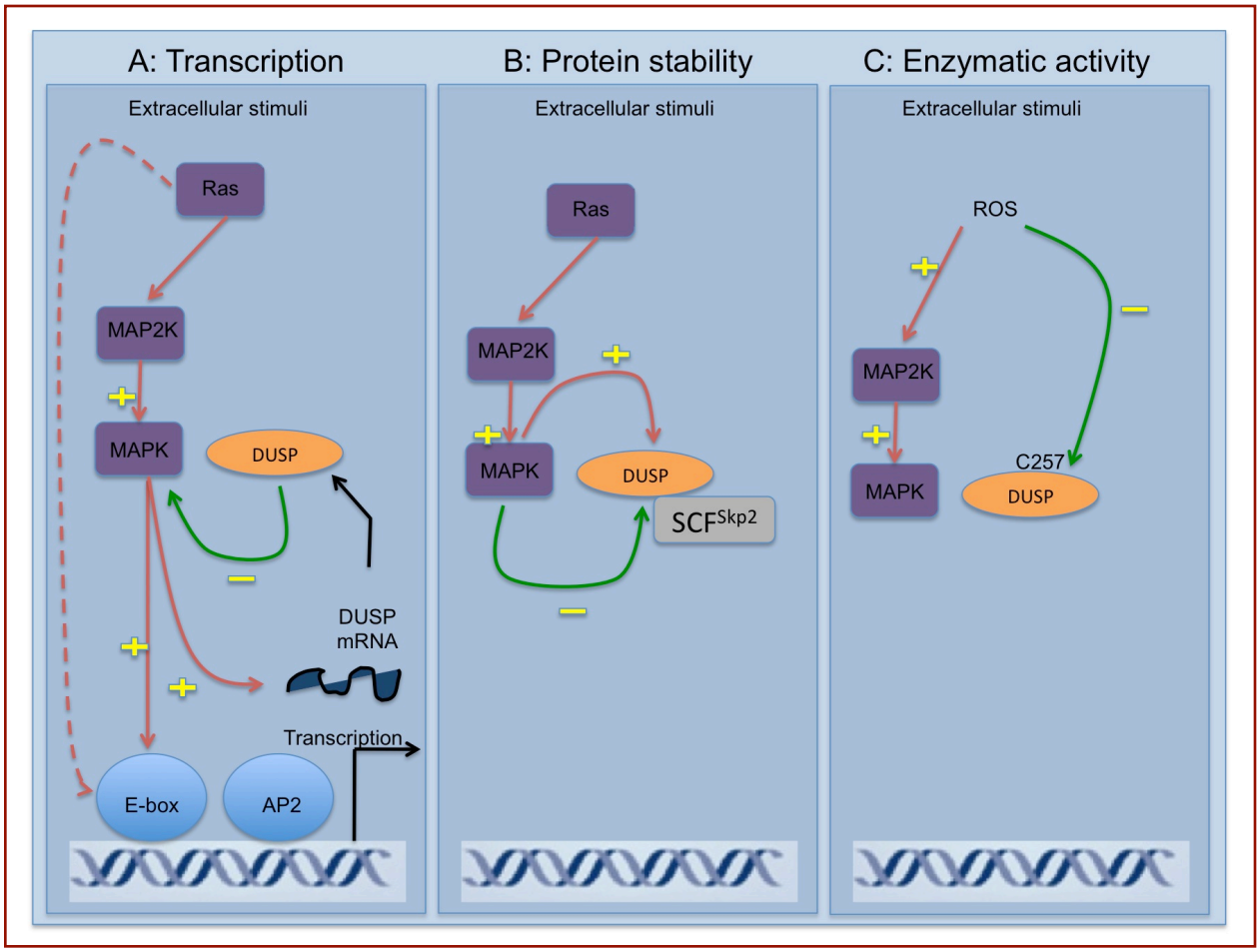


Figure 12 Three methods for regulating DUSP (MPK) activities

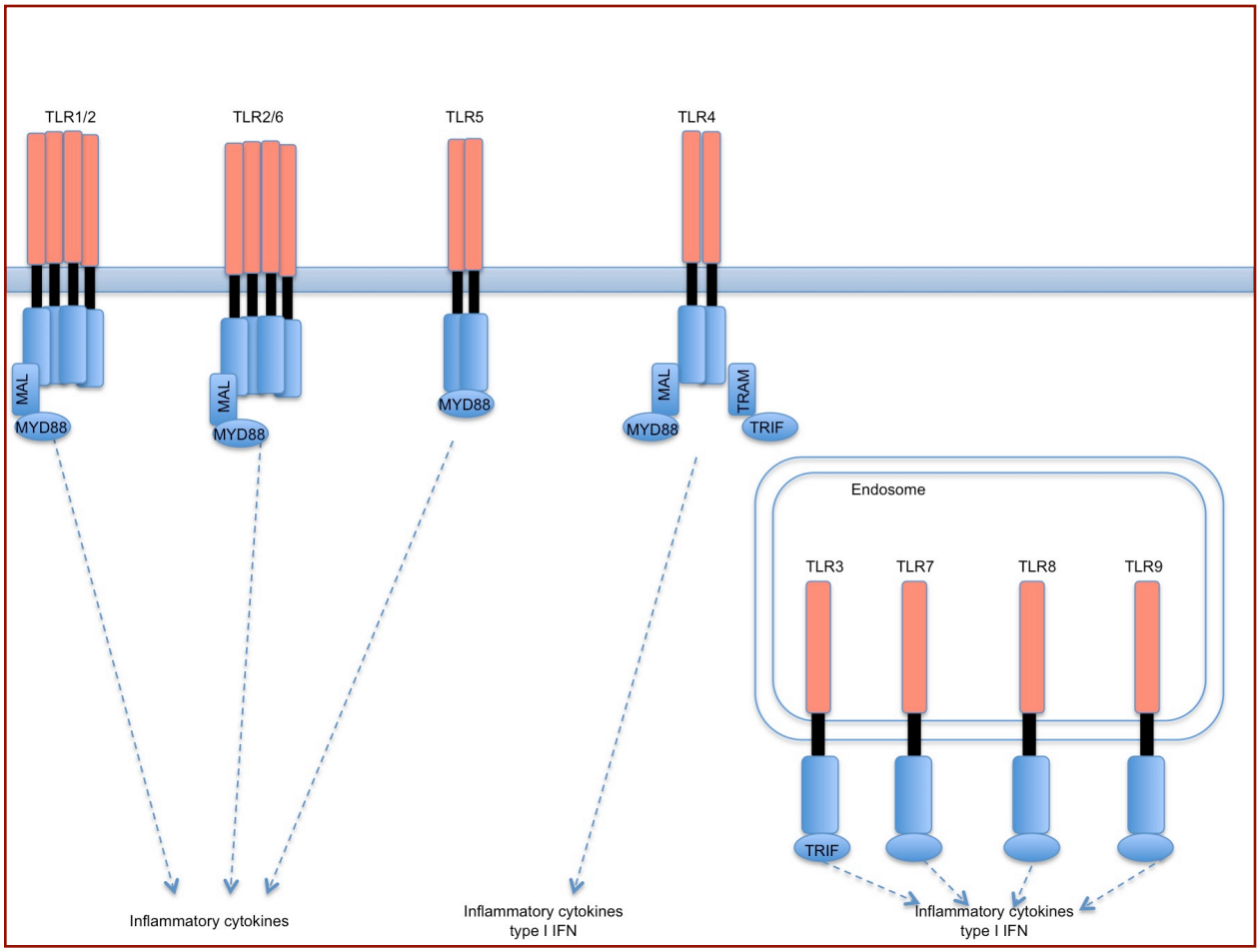


Figure **13 Adaptor molecule combinations signaling that allow for TLR specificity in signaling**

3.3.1 Cytosolic pattern recognition receptors

Cytosolic PRRs can be divided into retinoic acid-inducible gene I (RIGI)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Mogensen 2009). RIGI and MDA5 are two well-studied RLRs that function as RNA heilcases to sense cytoplasmic RNA by binding of dsRNA, 5'triphosphate RNA or self-derived small RNAs generated by RNase L to their C-terminal domain (Mogensen 2009).

NLRs belong to a family of innate immune receptors that have an N-terminal effector domain (downstream signaling), central nucleotide-binding NACHT/NAD domain (ligand induced self-oligomerization) and a C-terminal LLR domain (recognition of PAMPs) (Mogensen 2009; Rasmussen, Reinert et al. 2009). The NLRs can be further subdivided into the NODs and Nalps (NACHT, LLR and pyrin domain (PYD) containing) (Rasmussen, Reinert et al. 2009). NOD1 and NOD2 represent the two best-characterized NODs, whereas Napl1 and Nalp3 represent the two best-characterized Nalps (Mogensen 2009; Rasmussen, Reinert et al. 2009). Nalp1 and Nalp3 play an important role in assembly of the inflammasome complex, which is responsible for caspase-1 activation and the downstream production of IL-1β and IL-18 (Rasmussen, Reinert et al. 2009).

The interferon inducible dsRNA protein kinase, PKR, is another well-characterized cytosolic sensor. Upon binding to dsRNA, 5'triphosphate RNA or poly (I-C), a conformational change in PKR occurs that allows for its autophosohoylation, dimerization and subsequent phosphoryaltion of eukaryotic initiation translation factor alpha (eIF2α), for the inhibition of protein synthesis in the cell (Mogensen 2009).

Currently known cytoplasmic DNA sensors are DNA-dependent activator of IFN-regulatory factors (DAI), absent in melanoma-2 (AIM 2) and FI16 (an AIM 2-like receptor) (Rasmussen, Reinert et al. 2009; Unterholzner, Keating et al. 2010). DAI was the first identified cytosolic DNA sensor that has been shown to activate the IRF-family of transcription factors via its C-terminal region (Rasmussen, Reinert et al. 2009). DAI activation involves DAI binding to DNA in a length dependent manner and is independent of the form of the DNA (B or Z-form)

(Rasmussen, Reinert et al. 2009). AIM 2 and IFl16 are PYHIN proteins belonging to the PYHIN protein family that contains a pyrin domain and two DNA-binding HIN domains (Unterholzner, Keating et al. 2010). Upon sensing exogenous viral dsDNA, Aim 2 plays a role in inflammasome complex formation for the activation of caspase-1 and Fl16 plays a role in the induction of IFNβ via IRF3 and NFkB induction in addition to its regular role in cell proliferation and differentiation (Unterholzner, Keating et al. 2010).

Inflammasomes are considered caspase-1 activating molecular platforms that lead to the activation downstream production of proinflamatory cytokines (Schroder and Tschopp 2010). Caspases are cysteinie proteases whose activity is regulated by proteolytic cleavage and their activity can be divided into two categories: proinflammatory caspases (caspase-1, -11, -12) and pro-apoptotic caspases (caspase-1, -4, -5) (Schroder and Tschopp 2010). Caspase-1 is the most characterized proinflammatory caspase that plays a role in inflammasome complex formation. The inflammasome complex consists of self-oligimerization proteins such NLRs and AIM2 (Schroder and Tschopp 2010). The components (NLRP1, NLRP3 IPAF and AIM2) within the protein scaffold determine the activation mechanism of the inflammasome to mediate the caspase-1 dependent processing of the inflammatory cytokines (Schroder and Tschopp 2010).

3.4 Virus-mediated PRR

Viruses have many PAMPs that may be present on the infecting virion (such as viral glycoproteins) or may be produced during viral replication within the cell (such as nucleic acid structures DNA or RNA). In general, research has shown that TLRs 2, 3, 4, 7, 8 and 9 are able to detect nucleic acids or glycoproteins which are PAMPs associated with viral infection (Akira and

Takeda 2004; Christensen and Thomsen 2009). HSV-1 and HSV-2 infection is known to activate the innate immune response via TLR dependent pathways (TLR2, 3 and 9) and TLR independent pathways (RIG-I, DAI, PKR) (Zhu 2007; Daubeuf, Singh et al. 2009). Though the mechanism is not clearly understood, TLR2 has been shown to recognize HSV viral surface glycoprotein, and the recognition of HSV by TLR9 appears to be due to the unmethylated CpG motifs, though this recognition has been cell-type specific (Mogensen 2009). Cytosolic PRRs, such as RLRs, DAI and PKR, have also been shown to play a role in the recognition of HSV (Malmgaard, Melchjorsen et al. 2004; Rasmussen, Sorensen et al. 2007; Zhu 2007; Mogensen 2009)**.**

3.5 Adaptor molecules linking pattern recognition receptors (PRRs) to signaling cascades

As previously mentioned, upon TLR stimulation by ligand binding or the detection of PAMPs within the cytoplasm the induction of receptor dimerization and associated conformational changes that occur allow for cytosolic adaptor proteins to be recruited to their TLR TIR domains. The adaptor molecules, MyD88 and TIR domain-containing adaptor inducing IFN-β (TRIF), represent the two primary adaptors that are recruited to the TIR domains of TLRs (Akira, Uematsu et al. 2006; Zhong, Tien et al. 2006; Mogensen 2009; Rasmussen, Reinert et al. 2009) (Figure 14).

Examples of TLRs that use different adaptors to connect to signaling cascades are TLR3, TLR4 and TLR9 (Zhong, Tien et al. 2006). To activate the IkB kinases (IKKs), MAPKs, TLR3 utilizes the MyD88-independent and TRIF-dependent pathways; TLR4 utilizes the MyD88–dependent and TRIF-dependent pathways; and TLR9 utilizes the MyD88-dependent pathway (Zhong, Tien et al. 2006).

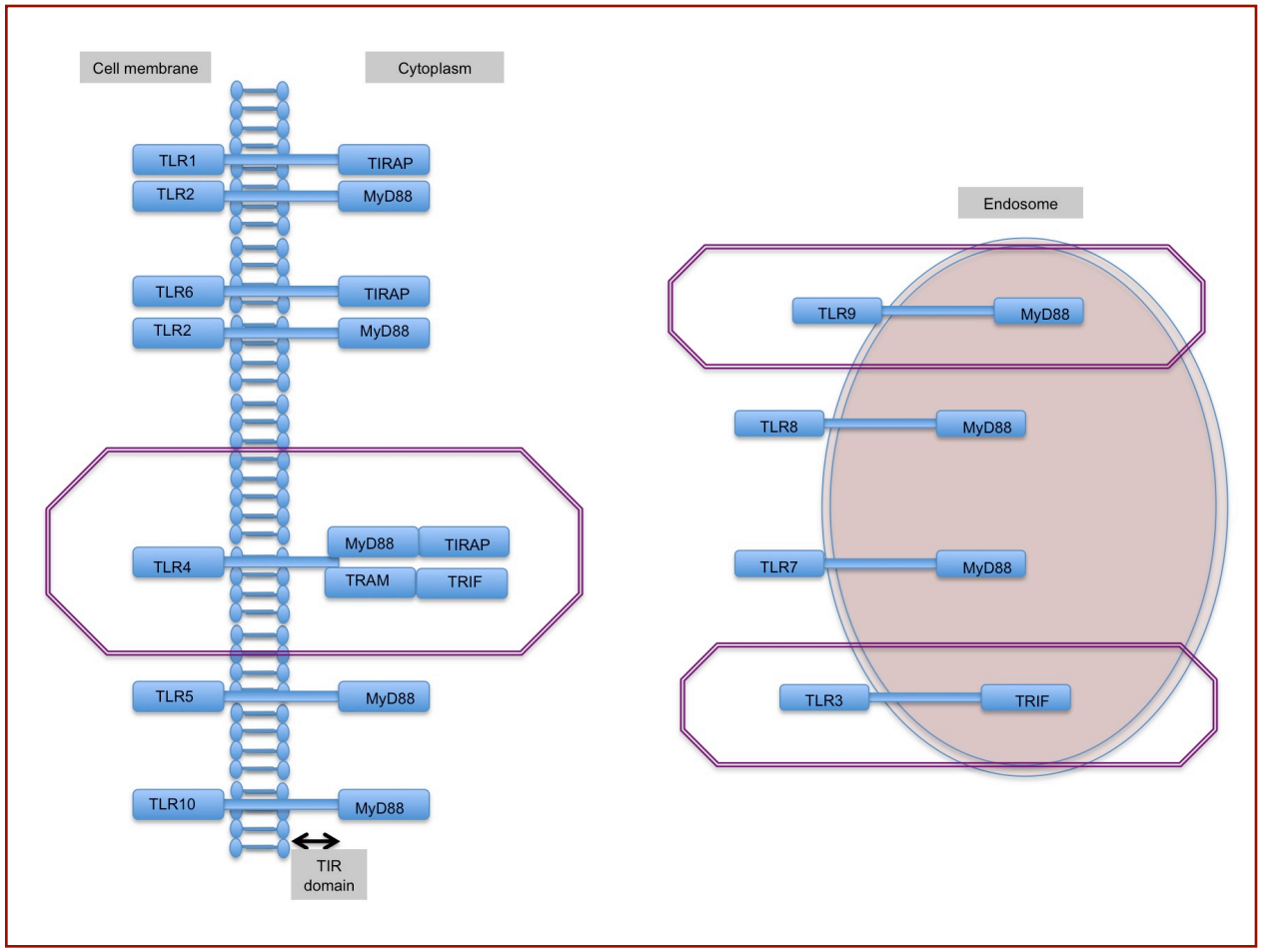


Figure 14. The two primary adaptor molecule responsible recruited to TLR TIR-domains.

The MyD88-dependent pathway involves the interaction of MyD88 with transforming growth factor-β-activated kinase (TAK1), TAK binding protein-1 (TAB1), TAK binding protein-2 (TAB2), and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Zhong, Tien et al. 2006). The TRIF-dependent pathway involves the interaction of TRAF family-member-associated NFkB activator (TANK) binding kinase-1 (TBK1) and TRAF6 to activate the IKKs, IRF3/7 and induce caspase 8 dependent apoptosis (Zhong, Tien et al. 2006).

Non-TLR dependent adaptor molecule virus-induced signaling adaptor (VISA) and TRAF6 are known to associate with PRRs within the cytoplasm such as the DNA sensor AIM2, and RIG-I-like receptors (RLRs) RIG-I and MDA5 (Konno, Yamamoto et al. 2009). Initially, VISA had been identified by four independent groups, and as a result VISA can also be referred to as interferon-β promoter stimulator 1 (IPS-1), mitochondrial antiviral signaling (MAVS) or Cardif (Kawai, Takahashi et al. 2005; Seth, Sun et al. 2005).

3.6 TNF receptor associated factor (TRAF) mediated signal transduction

TNF receptor-associated factors (TRAFs) are a family of evolutionarily conserved multi-functional adaptor proteins found in mammals that bind to surface pattern recognition receptors (PRRs) (Arch, Gedrich et al. 1998; Bradley and Pober 2001; Chung, Park et al. 2002). They physically and functionally connect cell PRRs to signaling transduction pathways by the recruitment of additional proteins from within the cytoplasm (Bradley and Pober 2001; Chung, Park et al. 2002). This leads to the formation of multiprotein signaling complexes on the cell surface and within the cytoplasm that are capable of initiating cellular responses associated with interconnected signaling cascades (Arch, Gedrich et al. 1998; Bradley and Pober 2001; Chung, Park et al. 2002).

All TRAF proteins have a carboxy-terminal (C-terminal) TRAF domain (Bradley and Pober 2001; Chung, Park et al. 2002). The N-terminal contains a RING finger with several zinc motifs, which is absent only in TRAF1 (Chung, Park et al. 2002). It has been shown that the intracellular location of TRAFs changes with PRR activation such that they are distributed throughout the cytoplasm or to intracellular punctate structures in resting cells (Bradley and Pober 2001;

Chung, Park et al. 2002). After PRR activation TRAFs can be rearranged within the plasma membrane, recruited to the membrane rafts within the plasma membrane, and recruited to the nucleus and perinuclear regions (Bradley and Pober 2001; Chung, Park et al. 2002). This rearrangement and recruitment is speculated to allow for stabilization of the receptor-adaptor complexes near to cellular proteins necessary for signal transduction, sustained signaling of the activated receptor and sequestering to regions to downregulate the signals being transduced (Bradley and Pober 2001; Chung, Park et al. 2002).

Currently there have been six functionally divergent TRAFs identified (TRAF1-6) that have been shown to participate in the regulation of adaptive and innate immunity, embryonic development, bone metabolism, cell survival, proliferation, differentiation and stress responses (Bradley and Pober 2001; Chung, Park et al. 2002).

TRAF1 is unique among the other TRAFs as it does not have an N-terminal ring finger and shows restricted tissue distribution. TRAF1 can interact with many TNFR family members, inclusive of herpes-virus entry mediator (HVEM), CD30 and CD40 for the activation of NFkB, JNK and the induction of apoptosis (Bradley and Pober 2001; Chung, Park et al. 2002).

TRAF2 has been extensively studied in terms of structure and function and is the most widely expressed TRAF in almost every tissue examined (Bradley and Pober 2001; Chung, Park et al. 2002). It has been shown to play a cytoprotective role, mediating signals for the activation of the transcription factors NFkB and activating protein-1 (AP-1) (Bradley and Pober 2001; Chung, Park et al. 2002).

TRAF3 has been shown to interact with members of the TNF receptor family members and to be important in T-cell dependent immune responses, NFkB activation and inhibition as well as in the induction of apoptosis (Bradley and Pober 2001; Chung, Park et al. 2002).

TRAF4 has been shown to be the only TRAF that localizes to the nucleus, and, unlike other TRAFs, it does not regulate signaling through cell surface receptors (Bradley and Pober 2001; Chung, Park et al. 2002). Though highly expressed during embryogenesis, TRAF4 has been shown to play a functional role in neural multipotent cells and in epithelial stem cells in adults (Bradley and Pober 2001; Chung, Park et al. 2002).

TRAF5 is functionally and structurally close to TRAF2 (Bradley and Pober 2001; Chung, Park et al. 2002). It has been shown to play a role in the activation of NFkB, AP-1 transcription factors and of CD40- and CD27-mediated lymphocytes (Bradley and Pober 2001; Chung, Park et al. 2002).

TRAF6, like TRAF2 and TRAF5, predominantly localizes to the membrane regions and contains a receptor binding specificity that is important for its role as a mediator of signaling through the TNF receptor family and the TLR family (Bradley and Pober 2001; Chung, Park et al. 2002). Functionally, TRAF6 is important for the activation of NFkB, JNK, and p38 as well as for CD40 mediated signaling (Bradley and Pober 2001; Chung, Park et al. 2002).

Pathogen sensing by PRRs that interact with functionally divergent adaptor proteins such as the TRAFs, function to mediate incoming signals (PAMPS) and recruit other proteins for the formation of signaling complexes. These complexes function as platforms for activation and regulation of downstream signaling pathways known to be involved in the innate immune re-

sponse. Suggesting that the different adaptors, and their combinations utilized by the membrane bound and cytosolic sensors are indispensable, and form a major part of the early cell sensing mechanisms within cells that function to detect pathogens and are tailored to influence specific pathways, some of which may overlap or allow for cross-talk to regulate the transcription of downstream genes.

The ability of TRAFs to serve as points of convergence from initial sensors of the cells, and as points of divergence for downstream signaling pathways, allows for an appropriate and tailored biological response to be mounted.

3.7 Major signaling pathways activated early in response to PRR recognition

The assembly of intracellular adaptor protein complexes after PRR engagement acts as a platform for the recruitment of other proteins involved in the activation of downstream signaling cascades via phosphorylation events, ubiquitination or protein-protein interactions to culminate with the activation of transcription factors involved in activating and regulating the innate immune response.

The main signaling cascades known to be activated after PRR-adaptor engagement are nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), mitogen-activated protein kinase (MAPK) and interferon regulatory factors (IFRs) (Bradley and Pober 2001; Chung, Park et al. 2002; Mogensen 2009). The activation of the MAPK and NFkB pathways allows for induction of a proinflammatory response involving the transcription factors activator protein-1 (AP-1) and NFkB, respectively. The activation of IRF pathway allows for the induction of IFN production

through binding to their cognate IRF-elements in promoter regions for IFN production (Mogensen 2009).

AP-1 activity is regulated by MAPKs (JNK and p38) via direct phosphorylation or through the transcription of AP-1 components such as cJun, Fos and activating transcription factor-2 (ATF2) (Chung, Park et al. 2002). The activation of NFkB can be through adaptor complex interaction with NIK, MEKK1 and MEKK3 whereas the activation of MAPKs can be through adaptor complex interaction with upstream MAPKs such as apoptosis signal-regulating kinase (ASK1) and MEKK1 (Chung, Park et al. 2002).

3.8 Mitogen-Activated Protein Kinase (MAPK) Cascade

As previously mentioned, the MAPK pathway consists of a three-tiered module involving sequential tyrosine and threonine phosphorylations of a MAP3 kinase (MAP3K) to a MAP2 kinase (MAP2K) to a MAP kinase (MAPK), which translocates to the nucleus and acts on transcription factors important in the innate immune response (Figure 15).

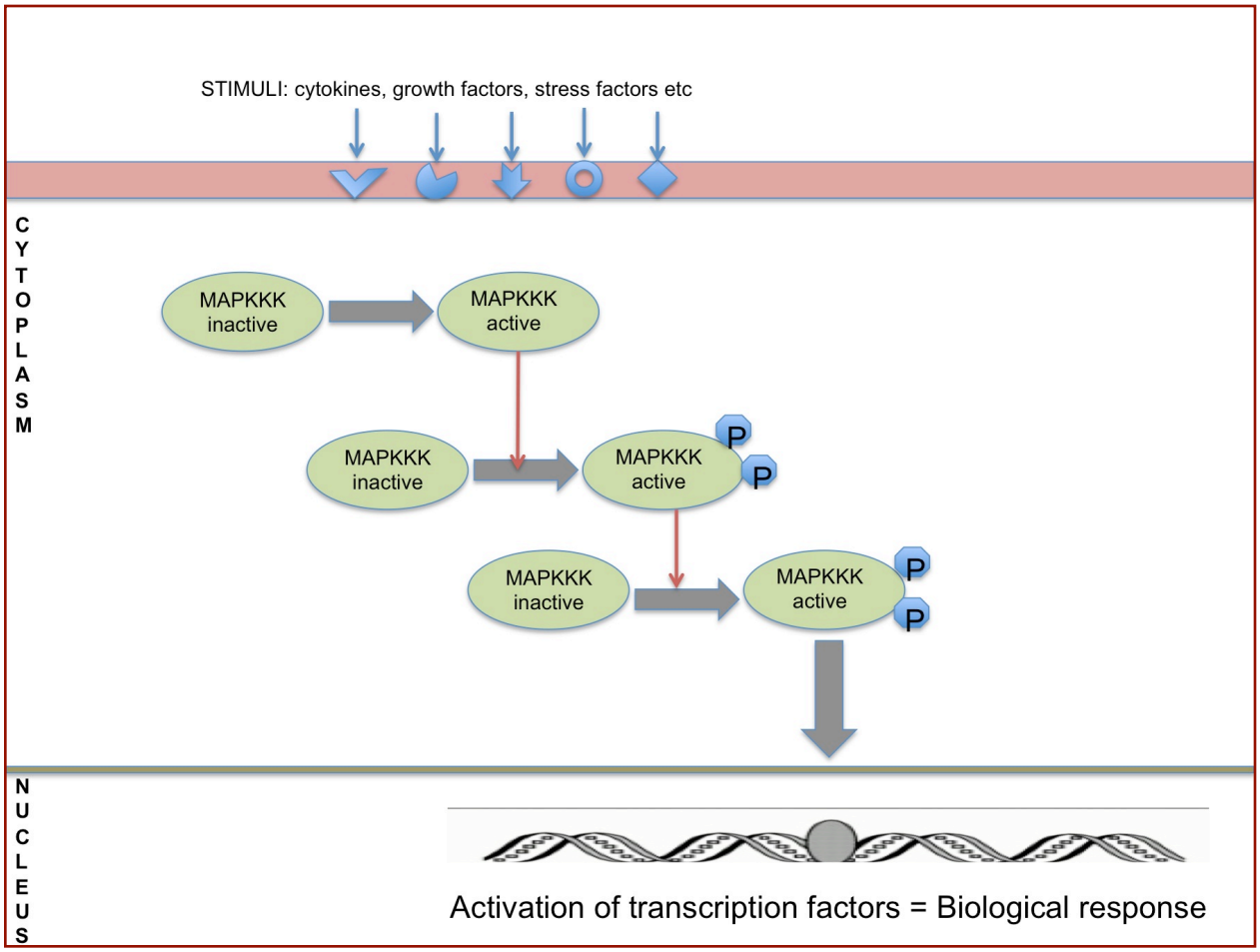


Figure 15. Three tiered module of MAPK activation

MAPKs can be divided into three broad categories: c-Jun NH2-terminal kinases (JNKs), p38 MAPK and extracellular signal-related kinases (ERKs). Commonly referred to as the mito-genic MAPKs, ERKs play a major role in cell division, migration and survival whereas JNK and p38 play important roles in regulating apoptosis, cell cycle control, and inflammatory gene tran-scription (Figure 16). JNK and p38 are commonly referred to as the stress associated protein kinases (SAPKs) as their induction was initially found to be stress associated (Hargett, McLean et al. 2005; Rincon and Davis 2009).

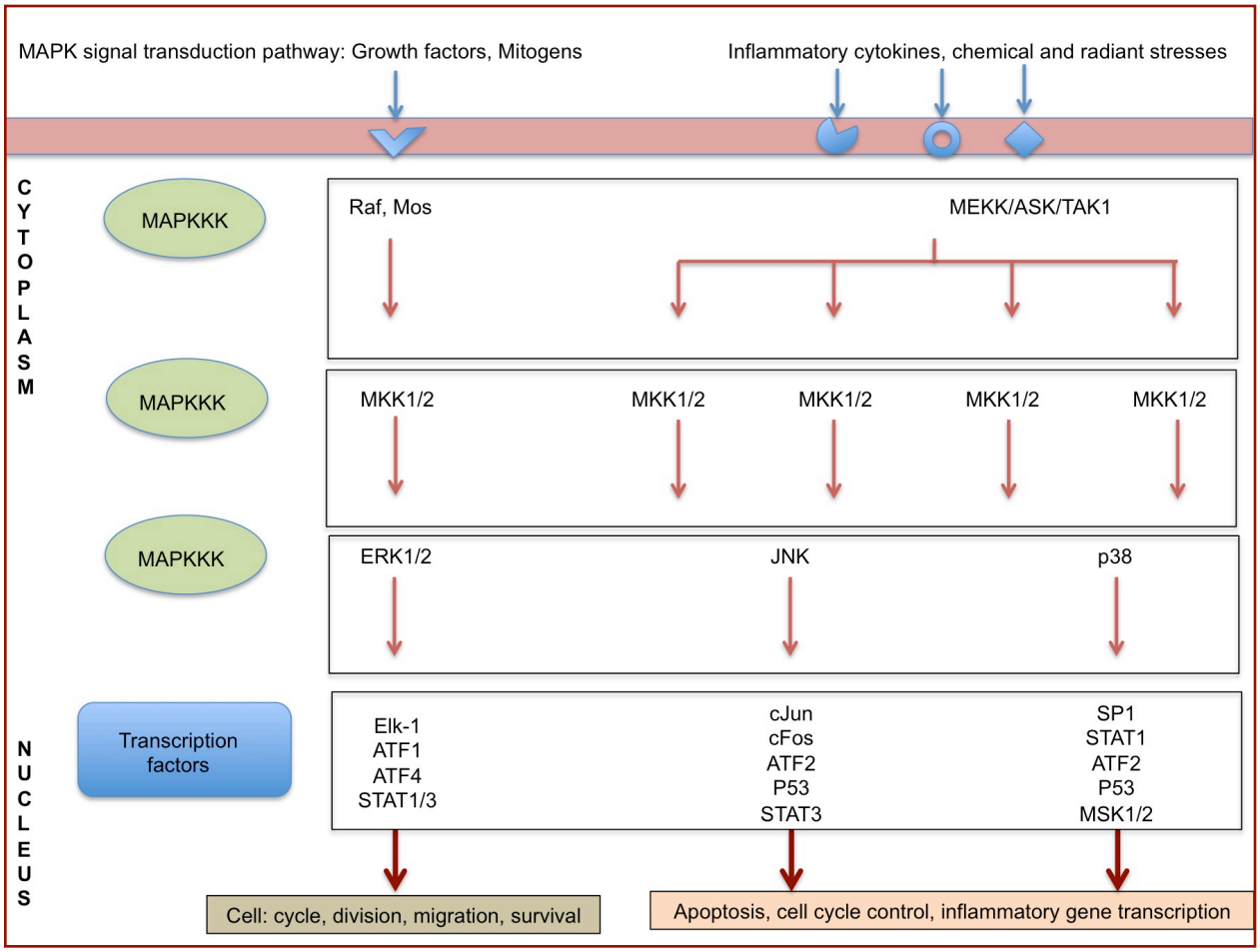


Figure 16. The three board categories of the MAPKs casacade

It should be noted that though the canonical method of MAPK activation involves sequential phosphorylation steps of a MAP3K to a MAP2K to a MAPK, MAP2K, an independent method of activation of MAPKs has been indentified and demonstrated for p38. This method involves p38 auto-phosphorylaton and subsequent activation via its interaction with the TAK1 binding protein TAB1 (Jiang, Gram et al. 1997; Ge, Gram et al. 2002; Tanno, Bassi et al. 2003).

JNK is encoded by three genes, JNK1, JNK2 and JNK3, that are alternatively spliced to give 10 isoforms, with JNK1 and JNK2 being ubiquitously expressed and JNK3 being primarily

expressed in brain, heart and testis (Bode and Dong 2007; Rincon and Davis 2009). The p38 gene can also be alternatively spliced to produce four isoforms: p38α (most expressed isoform), p38β, p38γ and p38δ (Rincon and Davis 2009).

JNK interacts with many cellular proteins such as Bcl-2 family member proteins, 14-3-3, MLK2, histone H3, c-Myc and JIP (Bode and Dong 2007). Transcription factors cJun, JunB, ATF2 and p53 are also known to be regulated by JNK (Bode and Dong 2007). JNK has been shown to interact hydrophobically with cJun in an ATP independent manner and when bound to JNK, cJun is targeted for ubiquitination and subsequent degradation (Fuchs, Xie et al. 1997). However, when cJun is serine phosphorylated by JNK it is protected from ubiquitination and its half-life increased (Fuchs, Dolan et al. 1996; Fuchs, Xie et al. 1997).

Mitogen-activated protein kinase kinase 4 (MKK4) and mitogen-activated protein kinase kinase 7 (MKK7) are MAP2Ks that activate JNK by phosohorylating JNK on a tyrosine and a threonine, respectively (Fuchs, Dolan et al. 1996; Bode and Dong 2007). Whereas JNK inactivation involves serine and tyrosine phosphatases and other dual specificity MAP kinase phosphatases (DUSPs/MKPs) (Bode and Dong 2007).

The state of JNK (active or inactive) along with its interaction with cellular proteins tends to dictate whether the associated protein is or is not protected from ubiquitination and subsequent degredation (Fuchs, Dolan et al. 1996; Fuchs, Xie et al. 1997). One of the previously mentioned transcription factors, p53 is a known regulator of apoptosis. All three isoforms of JNK have been shown to regulate p53 activity either by phosphorylation for sustained activation (active JNK) or by association with it for degradation (inactive JNK) (Fuchs, Adler et al. 1998;

Meylan, Burns et al. 2004). Though the association of JNK with p53 occurs in most cell types, the regulation of p53 has been shown to differ and sometimes to be opposing between JNK1 and JNK2 isoforms in certain cell types such as fibroblasts (Fuchs, Dolan et al. 1996). In this study they showed that though p53 was phosphorylated and activated under stress-induced conditions, JNK2 inhibition led to reduced p53 levels, whereas inhibition of JNK1 led to increased p53 levels and cells deficient in both isoforms led to no expression of p53 suggesting that cellular responses can be differentially regulated by the JNK isoforms (Fuchs, Dolan et al. 1996). JNK isoforms have also been shown to regulate the half-life and stability of cJun, such that JNK1 is more efficient in phosphorylating cJun, and JNK2 has a greater affinity for cJun thus targeting it for ubiquitination and subsequent degradation (Fuchs, Dolan et al. 1996).

Though the transcription factor cJun is not only a substrate for JNK but also for ERKs, protein kinase C (PKC), COOH-terminal Src kinase (CSK), DNA-dependent protein kinase (DNA-PK) and glycogen synthase kinase-3 (GSK-3), it may seem misleading that all functional models investigating JNK activity within cells look at the regulation of cJun (Bode and Dong 2007). However, because cJun activity is tied to AP1 activation and cJun is a major substrate of JNKs, the correlation of JNK activation and cJun transcription remains (Bode and Dong 2007).

JNKs themselves can be differentially regulated by their interaction with other proteins such as JNK interacting proteins (JIPs). JIPs function to inhibit the kinase activity of JNKs such that the phosohoryalted JNK is unable to activate downstream proteins that are influenced by phosphorylated JNK (pJNK) (Morrison and Davis 2003).

SAPK p38 activation has been linked to many cellular processes including inflammation, cell cycle control, apoptosis, senescence and tumorigenesis. Like JNK, p38 has been shown to be differentially expressed in varied cell types, such that p38α and p38β are the only ubiquitously expressed isoforms; p38γ is predominant in skeletal muscle and p38δ is predominant in lung, kidney, testis, pancreas and small intestine tissues (Ono and Han 2000; Ge, Gram et al. 2002; Tanno, Bassi et al. 2003). Many inflammatory diseases and apoptotic events have been linked to p38 activation, and it has been suggested that p38 plays a major role in the secretion of proinflammatory cytokines IL1β, TNFα and IL6 (Zarubin and Han 2005).

The MAPK known to selectively activate p38 are MKK3 and MKK6 (Ge, Gram et al. 2002; Tanno, Bassi et al. 2003). However, it has also been shown that MKK4, an upstream kinase for JNK activation, is capable of activating p38 in a cell-type specific manner and may thus serve as a site of integration of the p38 and JNK pathways (Jiang, Gram et al. 1997).

Though it was initially thought that activated p38 (phosphorylated p38) translocated into the nucleus, it has also been shown that inactivated p38 is present in both the cytoplasm and nucleus of cells and upon activation, translocation does occur, but activated p38 can also be found in the cytoplasm (Raingeaud, Gupta et al. 1995; Ben-Levy, Hooper et al. 1998).

There are few defined convergent and divergent points originating from the sites of incoming signal integration by PRRs and the downstream activation of MAPKs for specificity in the transcription factors activated through different upstream regulators and their combinations. Points of convergence are in the adaptor TRAF6, and points of divergence can be seen in

within the MAP3K where TAK1 and ASK1 are capable of activating both the p38 and JNK pathway (Figures 17 and 18) (Jiang, Gram et al. 1997; Morrison and Davis 2003).

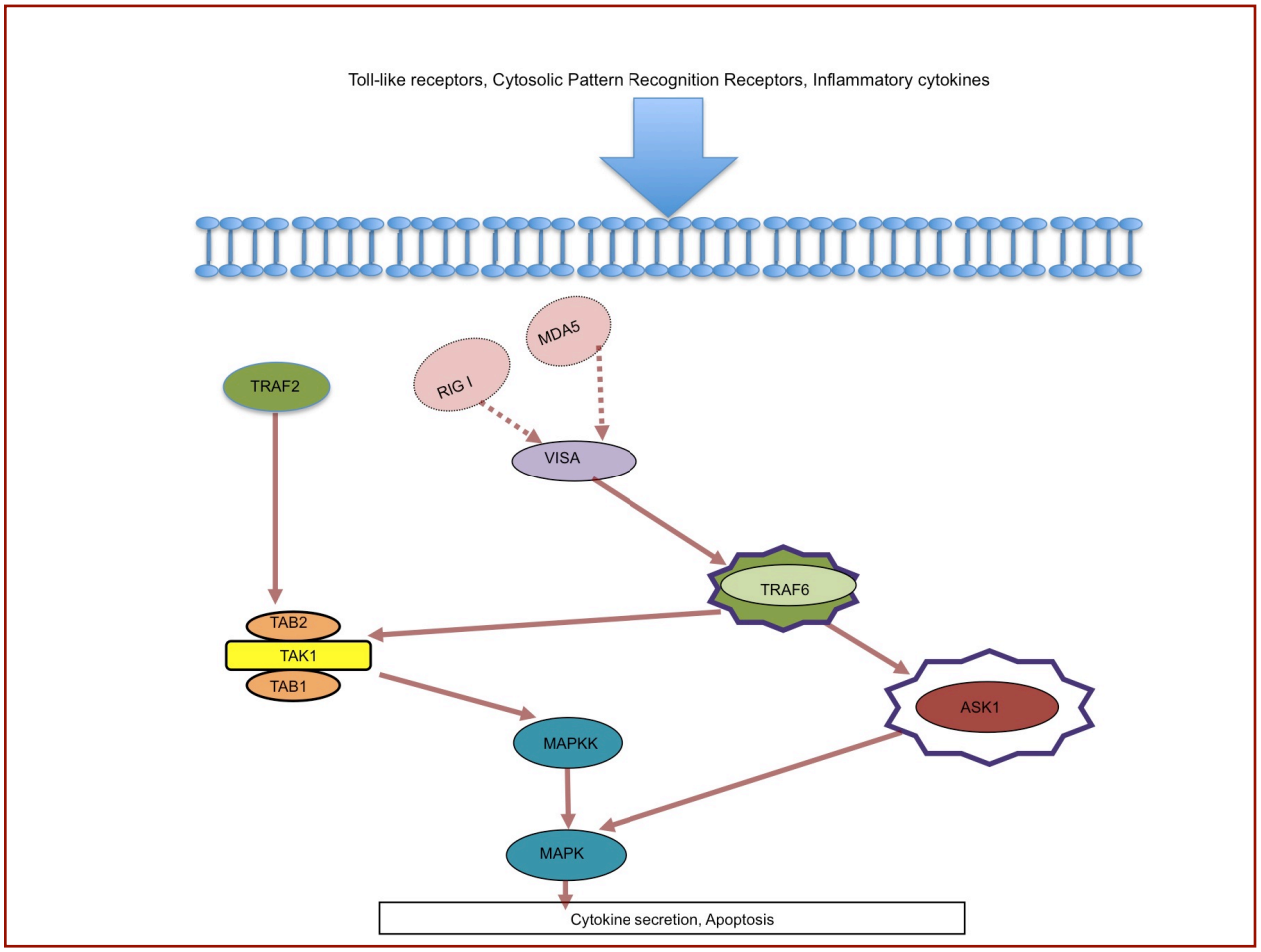


Figure 17. Points of convergence upstream of the MAPK pathway

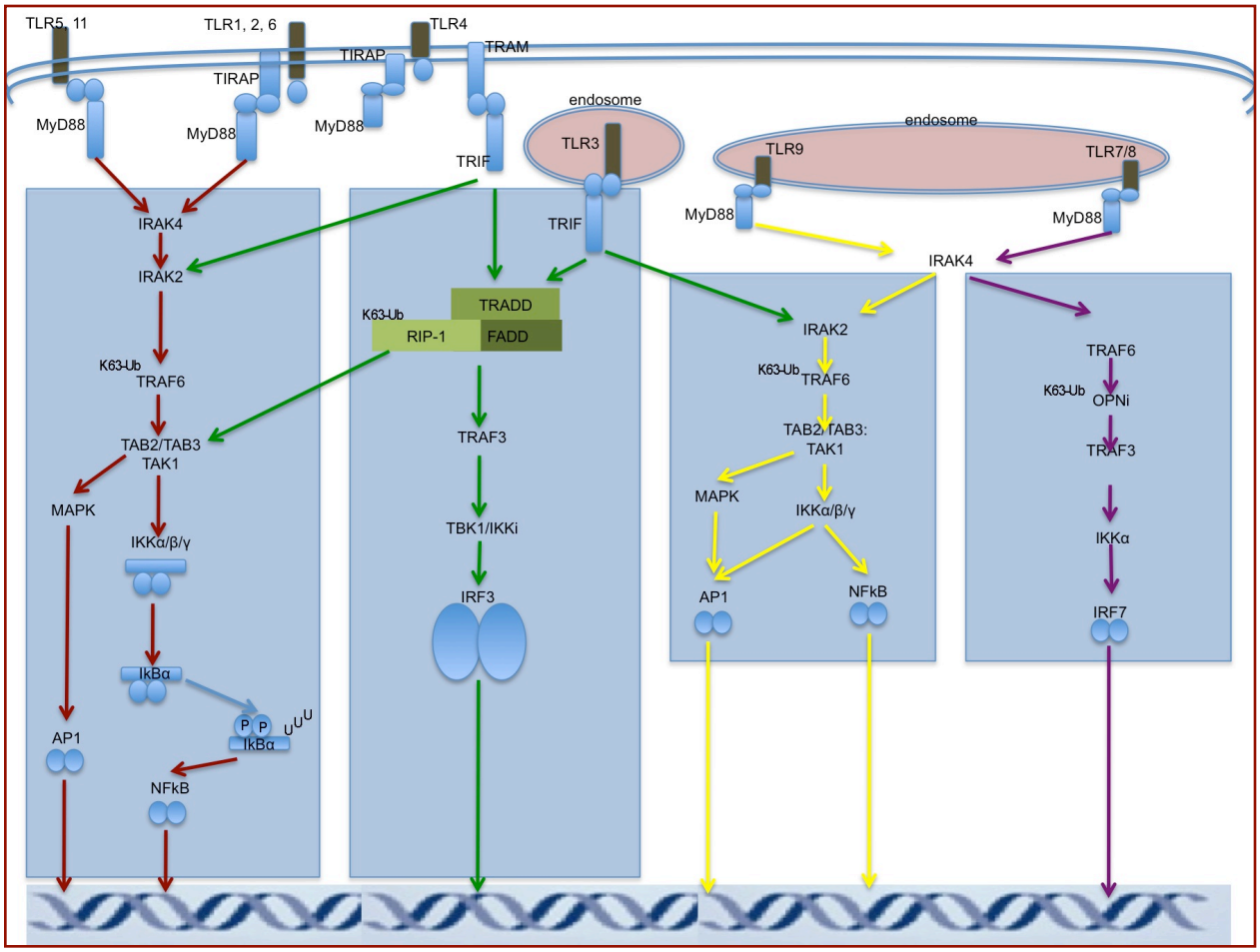


Figure 18. Points of divergence that allow for specific MAPK of transcription factors

MAPK p38 is has been shown to regulate MAP kinase-activated protein kinase 2 (MK2), MNK1, heat shock protein 27 (HSP27), cAMP-response element binding protein (CREB), ATF1/2/6, tristetraprolin (TTP), p53, MSK and nuclear factor of activated T cells (NFAT) (Ono and Han 2000; Zarubin and Han 2005). It should be noted that of the previously mentioned proteins and transcription factors regulated by p38, many of them play roles that can affect the host cell such that it is no longer a viable environment for virus replication (Ono and Han 2000; Zarubin and Han 2005). Examples include the following effects: TTP involvement in mRNA de-

stabilization, MNK1 role in translation initiation by phosphorylating eukaryotic initiation factor-4e (eIF4E), MSK1 phosphorylates pro-apoptotic protein Bad, and Sap-1a phosphorylation which is an important protein in the SRE dependent ternary complex factor (TCF) that is bound by transcription factor cfos, an AP1 component (Ono and Han 2000; Zarubin and Han 2005).

Dephosphorylation events are responsible for the control of MAPKs. The MAPK phosphatases (MKPs), also referred to as dual specificity MAPK phosphatases (DUSPs), function to dephosphorylate phosphothreonine and phosphotyrosine residues on activated MAPKs (Theodosiou and Ashworth 2002; Liu, Shepherd et al. 2007). Ten mammalian MKPs/DUSPs have been identified. They can be divided into two groups based on their subcellular location and method of transcriptional regulation and are regulated by three main mechanisms: transcription, protein stability and enzymatic activity (Table 2) (Theodosiou and Ashworth 2002; Liu, Shepherd et al. 2007).

Table 2 MAPK phosphatases (DUSP)

MKP	Species Orthologues	Substrate Specificity	Subcellular localization
MKP1	DUSP1, CL100, HVH1,3CH134, ERP	P38≈JNK>>ERK	Nuclear
MKP2	DUCP4, HVH2, TYP1	ERK≈JNK>>p38	Nuclear
MKP3	DUSP6, PYST1, RVH6	ERK>>JNK≈p38	Cytosolic
MKP4	DUSP9, PYST3	ERK>p38>JNK	Nuclear & cytosolic
MKP5	DUSP10	P38≈JNK>>ERK	Nuclear & cytosolic
MKP7	MKPM, DUSP16	JNK≈p38>>ERK	Cytosolic
MKPX	DUSP, B59, PYST2	ERK>>JNK≈p38	Cytosolic
DUSP2	PAC1	ERK≈p38>>JNK	Nuclear
HVH3	DUSP5, B23	ERK	Nuclear
HVH5	DUSP8, M3/M6	JNK≈p38>>ERK	Nuclear & cytosolic

MKP1, MKP2, DUSP2 and DUSP5 have been shown to localize primarily in the nuclear compartment, whereas MKP3-5 (i.e., DUSP 6, 9 and 10), MKP7, DUSP7 and DUSP8 are distributed within the cytoplasm and the nuclear compartment (Theodosiou and Ashworth 2002; Liu, Shepherd et al. 2007). It is thought that MKPs/DUSPs that are primarily localized to the nuclear compartments function as efficient regulators of MAPK and their activated transcription factors as they are in close proximity to the translocated (activated) MAPK and are readily induced by many of the same stimuli that activated the MAPKs (Figure 19) (Theodosiou and Ashworth 2002; Liu, Shepherd et al. 2007). Unlike the JNK isoforms, each of which is regulated by MPKs,

only the p38α and p38β forms are regulated by MKPs (Ono and Han 2000; Zarubin and Han 2005).

MKP1/DUSP1 is considered the prototype MKP and has been the most extensively studied (Theodosiou and Ashworth 2002). It has been shown that many downstream innate immune responses can be modulated by MKP regulation of MAPK activity and that MKP1 has a preference for regulating MAPKs p38 and JNK, thus playing an important role in the regulation of inflammatory cytokine biosynthesis (Theodosiou and Ashworth 2002).

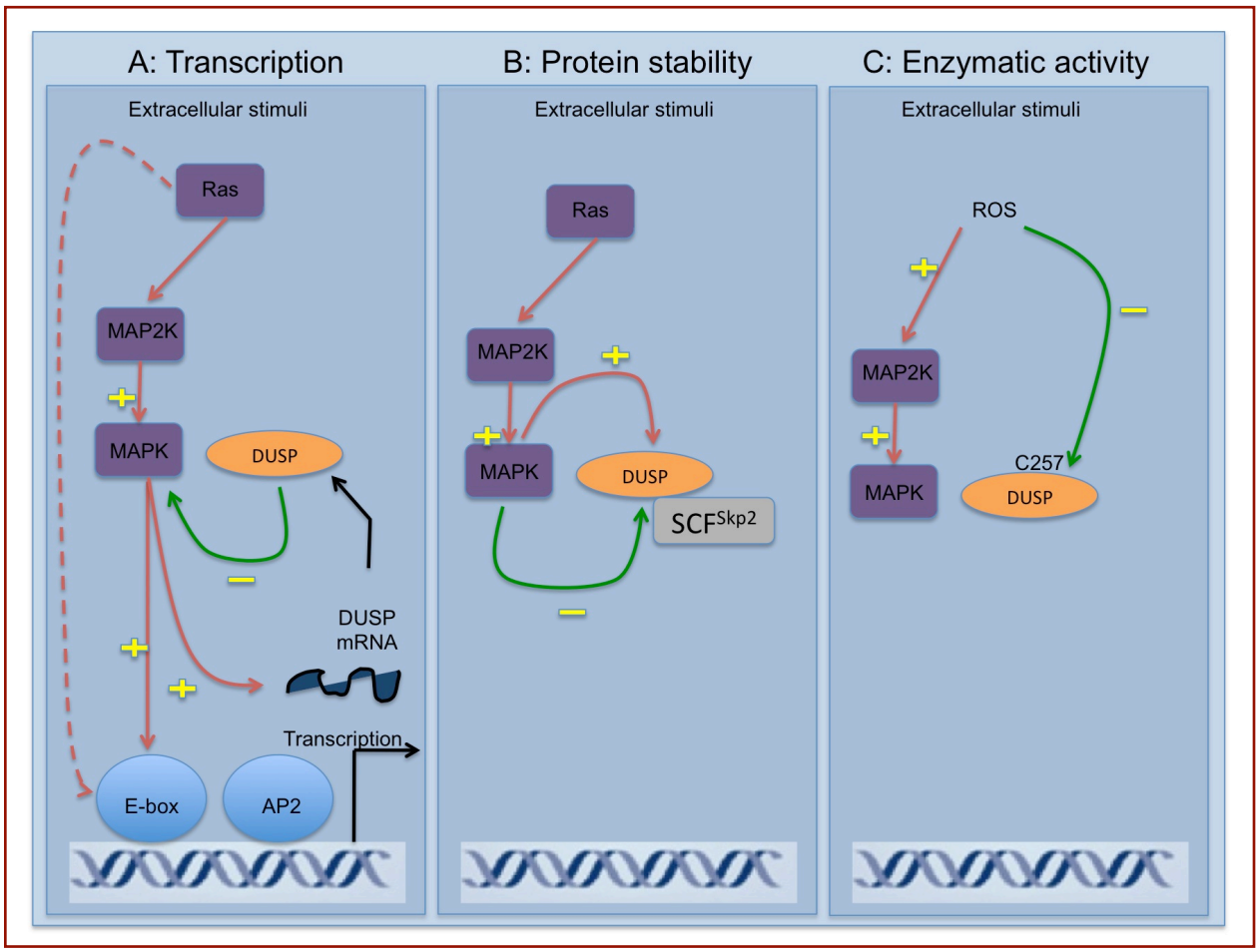


Figure 19. MAPK regulation by the MKP (DUSP)

3.9 MAPK-dependent apoptosis

As previously described, adaptor proteins TRAF2 and TRAF6 are known to directly activate or associate with factors that activate upstream MAP3Ks: TAK1 and ASK1. TAK1 and ASK1 lie at the top of the MAPK proinflammatory cascade. TAK1 associates with TAB1 and TAB2 to form a complex that activates downstream MAP2K for activation for the terminal MAPKS: p38 and JNK. ASK1 is also able to activate MAP2KS associated with both the p38 and JNK pathway, though its association has generally been with the JNK pathway.

Though the activation of p38 and JNK seems to have some redundancy, their pathways can be considered mutually exclusive for the control of defined cellular events, and they have been shown to be important mediators of intracellular signaling during innate responses such as inflammatory cytokine secretion and apoptosis.

This induction of apoptosis has been suggested to be an event that can be both MAPK dependent and MAPK independent, where the MAPK dependent induction is regulated by ASK1 (Ichijo, Nishida et al. 1997; Tibbles and Woodgett 1999; Saeki, Kobayashi et al. 2002; Takeda, Matsuzawa et al. 2003; Sumbayev and Yasinska 2006). ASK1 is a MAP3K that is ubiquitously expressed and can activate both the JNK and p38 pathways by phosphorylating (serine, threonine) their upstream MAP2Ks, MKK4/7 and MKK3/6, respectively (Sumbayev and Yasinska 2006). ASK1 has been found to play an important role in linking upstream TLR signals to the MAPK pathway, in skin innate immunity by its regulation of antimicrobial peptides, and in its interaction with PKR to promote apoptosis (Takizawa, Tatematsu et al. 2002; Sayama, Komatsuzawa et al. 2005; Li, Min et al. 2006; Sumbayev and Yasinska 2006). The MAP2K then phosphorylates

(threonine, tyrosine) JNK and p38, which in turn phosphorylate (serine, threonine) transcription factors cJun, cFos and ATF2, respectively (Sumbayev and Yasinska 2006). These transcription factors may form homodimers (cJun-cJun) or heterodimers (cJun-cFos, cJun-ATF2) and bind to the AP1 site (Halazonetis, Georgopoulos et al. 1988).

AP1 complexes are heterodimeric transcription factors that are ubiquitously expressed and bind a 12-O-tetradecanoylphorbol 13-acetate (TPA) response element (TRE) or a cyclic AMP response element (Karin, Liu et al. 1997; Xie, Pan et al. 2005). As with NFkB, consensus AP1 binding elements have been found in the promoter region of many *herpesvirus* genes (Xie, Pan et al. 2005). These complexes are made up of activating transcription factor 2 (ATF2), Jun (cJun, JunB and JunD) and Fos (cFos, FosB, Fra1 and Fra2) subfamilies all of which are modulated via phosphorylation (Karin 1995; Karin, Liu et al. 1997; Xie, Pan et al. 2005). These transcription factors are inducible within cells, and though low levels of cJun have been reported to be expressed in some cell lines, ATF2 is the only transcription factor (from the members listed above) that is known to be constitutively expressed (Karin 1995). Jun proteins can homodimerize or heterodimerize with the other factors to form stable AP1 binding factors, and it has been shown that Jun-Jun and Jun-Fos dimmers bind the TRE-responsive element and Jun-ATF2 bind cAMP-responsive elements preferentially (Karin, Liu et al. 1997).

Inactive JNK (nonphosphorylated) has been found to be associated with transcription factor p53 in non-stressed cells (Fuchs, Adler et al. 1998; Fuchs, Adler et al. 1998). The binding of JNK to p53 targets p53 for ubiquitination and subsequent degradation, whereas the activation of JNK (phosphoryaltion) releases p53 and allows for its stabilization within cells (Fuchs,

Adler et al. 1998; Fuchs, Adler et al. 1998). The disassociation of p53 from JNK and the subsequent transcription of AP1 dimers (cJun-cJun or cJun-ATF2) allows for sequential events of Bid cleavage, Bax translocation and cytochrome c release for the activation of caspase 3 and apoptosis (Figure 20) (Fuchs, Adler et al. 1998; Fuchs, Adler et al. 1998; Rahaus, Desloges et al. 2004).

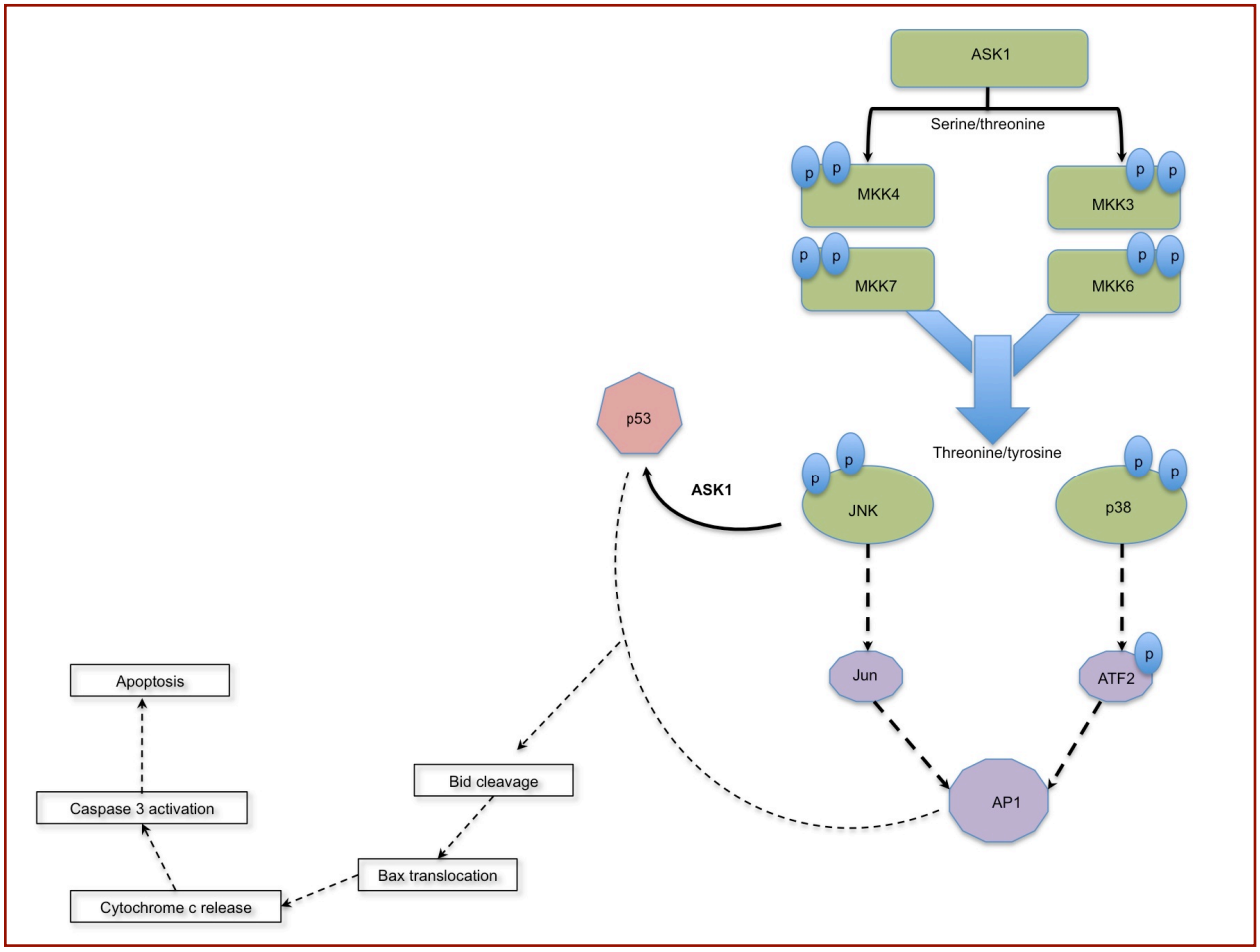


Figure 20. ASK-1-dependent apoptosis

The activation of executioner caspases 3, 6 and 7 has primarily been thought to be involved in apoptosis but they have also been shown to play a non-apoptotic role in inflamma-

tion, cell cycle control and cell differentiation and hematopoiesis (Creagh, Conroy et al. 2003; Best and Bloom 2004; Kuranaga and Miura 2007).

Regarding JNK it should be noted that all three isoforms of JNK have been shown to interact with p53 and contribute to p53 activation and stabilization (Buschmann, Potapova et al. 2001; Fogarty, Downer et al. 2003). It has also been shown that JNK1 and JNK2 can differentially regulate p53. In these studies, JNK1 expression led to decreased p53 levels, whereas JNK2 expression led to increased p53 levels, suggesting that pJNK1 was a negative regulator and pJNK2 was a positive regulator of p53 (Tafolla, Wang et al. 2005). Other studies have shown that dual inhibition of p38 and JNK also leads to increased levels of p53 dependent apoptosis, whereas studies also show that prolonged, rather than transient activation of JNK can lead to apoptosis possibly by inactivating suppressors of the mitochondrial death pathway (Lin and Dibling 2002; Lin 2003; Brown and Benchimol 2006).

Overall, it can be said that JNK seems to function pro-apoptotic under stress-induced conditions and anti-apoptotic under non-stressed conditions, suggesting that JNK's activity is both stimulus and cell type dependent.

Other important proteins for the regulation of p53 for MAPK-dependent apoptosis are the 14-3-3 proteins (Xing, Zhang et al. 2000). They are ubiquitously expressed in the cytoplasm of all mammalian cells and there are seven isoforms encoded: β, γ, ε, η, δ, θ and ζ (Shaw 2000; Xing, Zhang et al. 2000). They are known to bind phosphorylated Bad, a pro-apoptotic protein and sequester it in the cytoplasm, and that 14-3-3 phosphorylation by JNK allows for Bad-14-3-

3 dissociation followed by Bad translocation to the mitochondria for the induction of apoptosis (Sunayama, Tsuruta et al. 2005; Brown and Benchimol 2006).

Of the seven isoforms of 14-3-3, β and ζ are abundantly expressed in brain tissue; ζ and δ isoforms are phosphorylated by JNK; η and ζ have been shown to play a role in increasing the basal activity; δ has been implicated in the regulation of p53 transcription; and 14-3-3 levels have been correlated as an early predictor of CNS disease after SIV infection (Xing, Zhang et al. 2000; Helke, Queen et al. 2005; Hermeking 2005; Sunayama, Tsuruta et al. 2005; Tafolla, Wang et al. 2005).

3.10 Herpesvirus detection by PRRs and subversion of the downstream MAPK response

Herpesviruses are large enveloped DNA viruses whose replication cycle occurs in three waves for the expression of immediate early (IE or α), early (E or β) and late (L or γ) genes. There are 130 identified herpesviruses, eight of which are human pathogens (HSV-1, HSV-2, Human Cytomegalovirus (HCMV), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus 6 HHV, human herpesvirus (HHV-7) and human herpesvirus (HHV-8) (Vandevenne, Sadzot-Delvaux et al. 2010). Herpesviruses are known to modulate interferon effector functions, cytokine expression and activation, cytokine effector networks, chemokine effector networks, apoptosis and downstream adaptive immune responses.

Studies have shown that herpesviruses can be recognized by TLR-dependent mechanisms (TLR2, TLR3, TLR9) and by TLR-independent mechanisms (RIG-I, AIM2, DAI, PKR) (Zhu 2007; Daubeuf, Singh et al. 2009; Vandevenne, Sadzot-Delvaux et al. 2010).

Research has also shown that the activation of TLR2 via recognition of herpesvirus glycoproteins can be detrimental to the host, as TLR2 activation induces the expression of proinflammatory cytokines that can facilitate the development of lethal encephalitis (Kurt-Jones, Chan et al. 2004; Daubeuf, Singh et al. 2009; Vandevenne, Sadzot-Delvaux et al. 2010).

TLR3 has been shown to recognize the viral dsRNA generated through self-hybridization of viral genes transcribed from complementary DNA strands (Daubeuf, Singh et al. 2009). TLR3 has been shown to play a role in preventing herpesvirus spread to the central nervous system and its lack of activation has been shown to contribute to encephalitis (Zhang, Jouanguy et al. 2007; Daubeuf, Singh et al. 2009).

TLR9 has been shown to recognize the unmethylated CpG motifs in the DNA of herpesviruses (Kurt-Jones, Chan et al. 2004; Daubeuf, Singh et al. 2009; Vandevenne, Sadzot-Delvaux et al. 2010).

Herpesvirus DNA has been shown to serve as a template for cellular RNA Pol III, which functions to transcribe the dsDNA into RNA bearing 5'triphosphate moieties that is recognized by RIG-I (Chiu, Macmillan et al. 2009). Cytosolic dsDNA sensor AIM2 and DAI have been shown to recognize some herpesviruses to form the inflammasome complex for caspase-1 activation, and IRF expression for downstream IFN production and interferon stimulate gene (ISG) expression (Takaoka, Wang et al. 2007; Rathinam, Jiang et al. 2010). An overview of herpesvirus interference with the innate immune response is depicted in Table 3 and Figure 21.

Table 3. Herpesvirus proteins that interfere with the innate immune response

Herpesvirus	Viral Proteins	Cellular Targets
HSV-1	ICP34.5 ICP0 ICP27 Us11 Vhs	eIF2α, TBK1 IRF3 STAT1α OAS mRNA
VZV	Not known Not known Not known Unknown viral secretion factor	Jak2, STAT1α Golgi-cell surface trafficking NFkB pathway IFNα production
HCMV	Pp65 (ppUL83) IE86 Not known	IRF NFkB Jak1, p48
EBV	BZLF-1 LF2 BGLF4 BGLF1 LMP2A/LMP2B	IRF7 IRF7 IRF3 IRF3, IRF7 IFN receptors

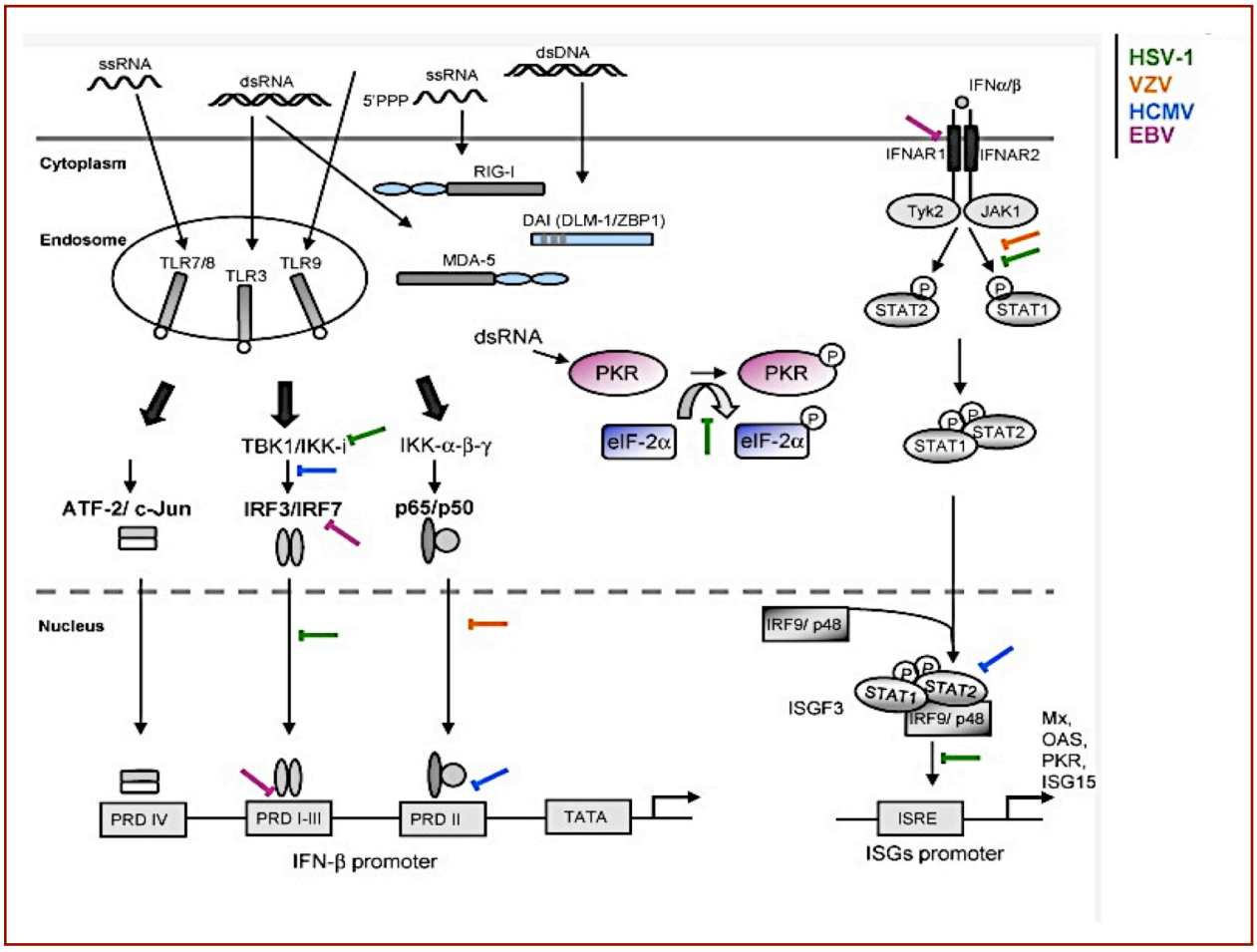


Figure 21. Diagram of known herpesvirus mechanisms of interference with the innate immune response at the cellular level.

In summary, we know that viral infection of host cells triggers not only early recognition sensors, but also an early stress response within cells. The endoplasmic reticulum is the site of folding and other post translation modifications for proteins, and it is also an essential organelle for viral replication and maturation (He 2006). Thus, viral infection can be considered a major cause of ER stress due to the many viral glycoproteins that are excessively produced (He 2006). It is therefore reasonable to hypothesize that after B virus infection the MAPK pathway and

downstream events associated with the MAPK pathway may differ in a host-dependent manner.

4 Specific Aim 3: Measure MAPK-induced gene products and proinflammatory mediators that are upregulated in response to MAPK-adaptor molecule activation to establish whether these events correlate with observed adaptive responses characterristic of each host species defenses against B virus infection.

4.1 Transcriptional and translational events regulated by the innate signaling pathways

The innate immune response involves the upregulation of genes (cytokine/chemokine and non-cytokine/chemokine) that lead to the downstream synthesis of proteins that influence the host response to infection and help shape the subsequent adaptive response. Initiation and propagation of the inflammatory response by cytokines must occur via one or more pathways that are activated after infection. The early upregulation of genes for the transcription of proinflammatory and immunosuppressive cytokines in response to viral infection can be done through four main pathways: the MAPK pathway, the IRF3/IRF7 pathway, the NFAT pathway and the NFkB pathway, each of which can occur concurrently or separately (Mogensen and Paludan 2001). The MAPK pathway has been shown to not only act at the transcriptional level for cytokine induction, but also to act at the posttranscriptional level where they functions in signal-induced stabilization of cytokines and chemokine mRNAs that contain A/U rich elements (Andrews and Matthews 2004).

Cytokines are proteins with potent activity that are bio-reactive at very low concentrations ranging from 10^{-10} to 10^{-13}ml/L (1ng to 1pg/ml) (Joost 2001). There are more than 100

identified cytokines, each of which exhibits unique structural, and some redundant, activities by its sharing of cellular receptors or receptor chains (Joost 1993). Cytokines can be divided into two main groups, although basic responses can be further broken down: T helper 1 cytokines (Th1) and T helper 2 cytokines (Th2).

Th1 response involves the secretion of cytokines that help shape the celluar immune response and activate cells associated with these defenses; Th2 response is involved in B cell activation and antibody development, both of which are part of the adaptive immune response. There is of course cross-talk between these two systems in that Th1 responses also induce antibodies and Th2 responses have cellular regulators. As such it has been shown that cytokines associated with the Th1 and Th2 responses can regulate each other such that IL2 a Th1 cytokine suppresses Th2 cytokine responses, and IL10 a Th2 cytokine suppresses Th1 cytokine responses (Mogensen and Paludan 2001; Sloan and Jerome 2007).

Regarding non-human primates such as macaques, comparative analysis of responses such as cytokine gene expression and secretion has been limited to human assays and reagents, especially as they have been shown to induce similar responses (Villinger, Brar et al. 1995). However, with the advent of software and molecular assays (arrays) specifically designed to look at genes and proteins such as cytokines involved in the innate immune response of non-human primates, a more thorough host-specific analysis of the roles played by specified transcription factors and cytokines in disease models can be made (Villinger, Brar et al. 1995).

4.2 Cytokines and chemokines involved in the modulation of innate components of the host response to infection

The induction of defined factors involved in immune responses leads to a coordinated response involving a plethora of cytokines and chemokines for downstream effector cells involved in the innate and adaptive immune response to be recruited and activated. The induction and secretion occurs in all cell types after stimulation but to differing levels or amounts. Though all cells are known to produce cytokines in response to stimuli, the repertoire of cytokines secreted by a cell depends on the type of stimulus, the duration, the intensity and the presence of other factors (Joost 2001). Cells which play primary roles in the innate response to infection and help shape the adaptive response, such as NK cells, dendritic cells and B cells, are expected to produce larger amounts (nanograms) of cytokines and chemokines than do cells of non-hematopoietic origin, such as fibroblasts and epithelial cells, which produce amounts that can be measured in picograms (Joost 2001).

Traditionally, when investigating cytokines involved in the innate immune response, one speaks of the proinflammatory cytokines. Some of the major proinflammatory cytokines are IL1, TNFα, IL6, IL12, GM-CSF and IFNγ (Joost 2001). However, IL2, LT, IL4 and IL10 are also considered proinflammatory (Joost 2001). Generally when one speaks of cytokines they are referring to the proinflammatory cytokines, but it must be noted that there are also cytokines referred to as anti-inflammatory cytokines, which act as immunomodulatory mediators of the inflammatory response and function to regulate proinflammatory activities. Some commonly known anti-inflammatory cytokines known to limit the magnitude and extent of inflammation are IL1Ra, IL4, IL6 IL10, IL11, IL13 and TGFβ (Opal and DePalo 2000; Joost 2001).

In general, it can be said during the regulation and modulation of host innate immune responses, there is a constant shift in the balance and regulation of cytokines such that their net effect is dependent on the time of release/secretion, the location of the effect, the responsiveness of cells within the area of release, the density of receptors for the region of its effect and the overall composition of other competing or synergistic cytokines within the defined area (Opal and DePalo 2000). Thus, it is important to consider all of these factors when looking at models of infection and disease and to be aware of the limitations of in vitro work as well as to be cautious in making generalized assumptions about *in vivo* studies.

4.3 Cytokine and chemokine response to herpesvirus infections

After infection, most herpesviruses have been shown to induce a plethora of biphasic cytokines responses: the first phase including IL1β, IL2, IL6, IL10, IL12, Il13, TNFα, IFNα/β, IFNγ and GM-CSF; and the second phase including cytokines IL8, MIP1α, MIP1β, MCP1 and RANTES (Mogensen and Paludan 2001). These responses are thought to be initially dependent on viral surface and tegument proteins, to be followed by a later response induced by factors involved in the replication of the virus (Sloan and Jerome 2007). The Th1 response to infection allows for early recognition, containment and removal of virus infection and it is for this reason that HSV-1 has found mechanisms to inhibit Th1 cytokine synthesis and upregulate the anti-inflammatory cytokine IL10 to inhibit the proinflammatory response (Sloan and Jerome 2007).

Because many early innate immune events that occur after B virus infection can be influenced by the MAPK pathway, it is important to investigate the transcriptional profile of factors

regulated selectively by each arm (p38 and JNK) of the MAPK pathway, and to investigate the induced cytokine profile in cells derived from both hosts.

In summary, the innate responses engaged in macaque and humans cells would be predicted to differ based on the observed differences in adaptive immune defenses induced in each species. Correlating these innate responses with adaptive defense outcomes of species specific B virus infections has not previously been possible. With the novel cell-based model of infection developed as part of this dissertation research, we can evaluate for the first time whether macaque and human cells do indeed differ in their innate defense strategies when infected with B virus and whether the observed responses correlate with the reported differences in adaptive immune responses observed in macaques and humans.

5 Materials and Methods

5.1 Cell lines

Macaque biopsies from male and female genital, skin and ganglion were obtained through a tissue-sharing program with Yerkes Primate Center, Emory University, Atlanta, GA in April, July and August 2007. The primary isolation and subculture of keratinocytes and fibroblasts and glial cells were then done using the different sections from the macaque tissue sections (skin, foreskin, clitoral hood and ganglion) received.

Briefly, tissue was removed from collection vessels and any accompanying media sterilely aspirated and blotted. If hair was present, it was removed with Scotch tape © and a razor. Scotch tape © was also used to remove the upper-most cornified epithelial layer (i.e., the top

layer of the skin). Tissues were then disinfected with an antiseptic or antimicrobial cleanser and rinsed in 30 mM HEPES buffer or phosphate buffered solution (PBS) (without Ca^{2+} and Mg^{2+}). The epidermal side was placed facing downward, rinsed and the buffer was replace with serum-free basal media. All fat and excess subcutaneous connective tissue were removed prior to cutting tissue into 2 mm^3-5 mm^3 pieces and placing these pieces into collagenase-dispase or trypsin-EDTA solutions overnight at 4°C for dissociation and digestion of layers.

Following overnight incubation for digestion, dermal and epidermal layers were separated and placed into a trypsin-EDTA solution at 37°C with gentle agitation for further digestion of the separated layers. Neutralization of digestion with serum was followed by dissociation of cells into suspension and by straining to remove all unwanted components of the respective layer. Collected cell suspensions were centrifuged at 180g and the supernatant was discarded. The pellet was resuspended in differing groups of defined media for each stated cell type (glial, keratinocyte or fibroblast) supplemented with Penicillin/Streptomycin/Amphotericin B solution or a Gentamicin/Amphotericin B solution. Note that for each cell type, several different media cocktails were used with varying concentrations of fetal bovine serum (FBS), human keratinocyte growth serum (HKGS) and antibiotic/antimycotic because it was not known which cocktail could support macaque primary cell line growth more efficiently. For each group, viable cell counts were then done using trypan blue (classical method for determining cell count), and cells were plated onto collagen coated T25 flasks and 6-well plates to establish a viable cell culture. Cells were observed every 24 hrs and media was replaced every 48 hrs. When cells were at an 80% density in their respective container they were subcultured. Primary cells were never allowed to become completely confluent and harvesting and cryopreservation of cells were

done as described for human keratinocytes and human fibroblasts (Current Protocols Unit 2.1). Glial cells were not passaged. They were removed and stored in liquid nitrogen for future use.

Human foreskin fibroblast (HFF) (ATCC, CRL-2097) cells were purchased from ATCC and grown in minimum essential medium (MEM) (Mediatech, Herndon, VA) supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 18% FBS. These fibroblasts are primary human foreskin fibroblasts (HFF) and are the fibroblast cell type used for current and past investigations in this lab to study B virus infection.

Human Epidermal Keratinocyte (HEK) cells were grown in EpiLife cell culture media (Invitrogen) supplemented with human keratinocyte growth supplement media (Invitrogen).

Vero African Green monkey kidney) cells (ATCC, CL81) were grown in Dulbecco's Modified Eagles Essential Medium (Cellgro) supplemented with 10% FBS

5.2 Viruses

The viruses used in this study: B virus laboratory strain E2490 propagated in Vero cells, HSV-1 MacIntyre (ATCC, VR-539) propagated in Vero cells and HSV-1 F strain (initially received from B. Roizman, The University of Chicago, Chicago IL) propagated in Vero cells.

All infections with virus were performed under BSL-4 conditions in a registered facility under Select Agent status in accordance with the Biosafety in Microbiological and Biomedical Laboratories (BMBL) manual (CDC/Office of Safety 2009). Though HSV-1 is not classified as a select agent, all infections were also performed under BSL-4 conditions unless noted differently in order to maximize the comparative analyses.

5.3 Subculture of cells (passaging), seeding of cells for experiments and cryopreservation

Briefly, for stated cell types, flasks and wells were washed twice with phosphate buffered saline (PBS), followed by the addition of Trypsin-EDTA. Vessels were then placed in an incubator at 37°C for a few minutes for cells to round up and detach. After detachment, the trypsin was neutralized by the addition of serum-containing media and cells were resuspended in defined media with or without antibiotic/antimycotic for the stated cell type and placed into new flasks or seeded into well of plates. For all experiments aliquots of each cell suspension was counted via the classical method involving trypan blue staining and counting with hemocytometers. They plates, flasks or LabTek™ chamber slides were seeded equally with each cell type. If cells were to be stored, then suspensions were transferred to sterile conicals and spun at 1200 RPM for 3-5 mins. Supernatants were discarded and pellets were resuspended in defined freezing media (10%DMSO with 20%FBS in DMEM). Cell counts were done manually using a hemacytometer and tubes/aliquots stored with a defined amount of cells. Aliquots were initially cooled and frozen at -80°C in a designated cell-freezing container followed by long-term storage in liquid nitrogen.

5.4 Detection of potential contaminants

Cell cultures maintained for a minimum of two days. Media and cells were collected separately and mycoplasm detection done according to protocol outlined by mycoplasma PCR detection kit (Sigma MP0035). Briefly, the kits contains a primer set specific to the highly conserved 16SrRNA coding region in the mycoplsma genome, pre-coated with appropriate dNTPs and primers in reaction tubes and internal DNA control (481bp). After PCR is performed, samples are run in 1.2 percent agarose gels. Cells were also grown and immunofluorescencence

staining was done using Hoechst to confirm the absence of mycoplasma (Battaglia, Pozzi et al. 1994; Valladeau and Saeland 2005).

For other potential contaminants, subculturing procedures were done and cells left for a minimum of two days in the absence of antibiotic or antimycotic in their respective media mixtures and visualized.

5.5 Determination of efficiency of plating

HFF, RMF, HEK and Vero cells were counted and seeded at a desired density equally for each cell line to be ready (85-90 percent confluent) in 2-3 days for infection in 12-well flat-bottom plates. Viruses used were HSV-1 (HSV-1 McIntyre and F strains) and B virus (E2490). Serial dilutions (10^{-1} – 10^{-6}) for each defined plaque forming unit (p.f.u.) stock virus was prepared in Hanks Balanced Salt solution (HBSS). Media was removed, cells washed with Phosphate Buffered Solution (PBS) twice. This was followed by the addition of 300ul of the respective dilution into each well, which was left for 1hour absorption at 37C. After 1 hour unadsorbed virus was removed and addition/replacement with a 1 percent methyl cellulose, 2 percent fetal bovine serum (FBS) mix and left for 48 hours (2dys). After 2 dys, infection was stopped by fixation with 100 percent methanol and monolayers stained with crystal violet or by immunofluroescense for the detection and the observation of plaques. The differing titers (pfu) calculated for each virus in the defined cell line were then used to calculate fold difference when compared to Vero cells titrated with that virus.

5.6 Infections

Cells were counted and seeded at a desired density equally for each cell line to be ready (85-90 percent confluent) in 2-3 days for infection. The following virus used for infections as outline by the experiments was B virus (E2490). The multiplicity of infection for all experiments was MOI of 5. The control was mock cell lysate (MCL) for infections unless otherwise stated. Mock cell lysate represents the Vero cell lysate from mock infected cells prepared, collected, and treated at the same time and same manner as the B virus stock that was being prepared. Mock cell lysate was used as the control for theses signaling studies as virus stock preparation is done using infected Vero cells and as such with the prepared virus stock, it will have components that are part of the lysed Vero cells and components that were secreted in response to B virus infection. Thus, MCL when compared to B virus stock should comprise of the same components minus virus, virus related or induced components.

Note: Calculated volumes of virus for an MOI 5 infection in the respective cell line (determined from the plating efficiency results) were placed into HBSS to give the working virus mixture.

For all time point experiments except those involving collection of supernatants for cytokine and chemokine analysis, media was removed and kept in 50ml conicals for the respective cell line. This media is referred to as spent media. Prior to infection media was removed, cells washed with Phosphate Buffered Solution (PBS) twice and experiments done in T25 flasks received 1ml virus mixture, 12 well plates received 300ul virus mixture per well, those done in 6 well plated received 500 ul per well and those done in LabTek™ chamber slides received 100-

300ul per. After virus input the flask/plate or chamber slides were placed at 37°C for 1hr for virus to absorb. After 1 hour absorption the unadsorbed virus was removed and well replaced with spent media (3ml per/T25 flask; 2ml/well of 6-well plate; 1ml/well of 12-well plate and 0.5-1ml per well for LabTek™ chambers).

Only in the cases where supernatants were being collected for cytokine analysis the replacement media after virus absorption was 2%FBS media and the input volumes were as follows: 500ul/well of 6-well plate and 300ul/well of 12-well plate. Note for all experiments that 1 hr post infection (pi) refers to the amount of time after virus had been placed onto cells (the 1 hr absorption period), whereas 1 hr post absorption (pa) refers to the amount of time after the unadsorbed virus was removed and the replacement media was added. All time points represented in the cytokine and chemokine analysis are representative of times post adsorption.

5.7 Immunofluoresence

Rhesus macaque fibroblasts (RMF), human foreskin fibroblasts (HFF), human epidermal keratinocytes (HEK) and African Green monkey kidney cells (Veros) were seeded onto LabTek™ chamber slides and/or into 6-well and 12-well plates. After no treatment or stated treatment (infection or mock infection), cells were fixed and permeabilized using 4% paraformaldehyde with Triton X or 100% methanol (MeOH). The addition of triton X or the use of MeOH disrupted cellular membranes such that staining on cytoskeletal components could be done. Cells were washed with PBS and incubated in 20% normal goat serum (NGS). This process was followed by PBS washes and subsequent incubation of the respective primary antibody (1°Ab) at a defined dilution in 10% NGS. Primary antibody incubation was followed by subsequent washes, incuba-

tion in appropriate fluorescent conjugated secondary antibody (2°Ab) at stated dilution in 10% NGS and additional washes with PBS. After the 2°Ab incubation and washes, other commercially available stains for DNA (Hoechst/DAPI), lectin membranes (Alexa Fluor 529), Vimentin (R28) and F-actin (Alexa Fluor 568 phalloidin) were added together, where applicable, and followed by PBS washes. Slides were then mounted, sealed and stored in a dark container at 4°C before and after viewing under Zeiss LSM 700 immunfluroescence scope. Plates were covered with foil and stored at 4°C before and after viewing under Zeiss LSM 700 immunfluroescence scope.

5.8 Polymerase chain reaction (PCR)

At stated times, post infection samples were collected with RTL buffer and purified according to manufacturer's instructions (RNeasy Mini kit - Qiagen). Concentration of purified RNA was determined using the Nanovue machine (GE). Purified RNA was then converted to cDNA using RT2 First strand kit (SA Biosciences) and PCR performed using RT2 SybrGreen/ROX qPCR mix kit in conjunction with one of the following: RT2 Profiler PCR Array Human Inflammatory Cytokines and Receptors array, RT2 Profiler PCR Array Human MAP Kinase Signaling Pathway array's (SA Biosciences) or self-designed primers that were ordered from Integrated DNA Technologies (IDT) to conserved regions in both human and macaque cells. Samples were run using the ABI 7900HT Real time PCR machine (SA Biosciences).

5.9 SDS Page and Western Blotting

After infection or mock-infection total cell lysates were collected using Laemmli buffer and aliquoted prior to storage at -20°C. Then 10% SDS gels were prepared and used for all pro-

teins analyzed. Gels were transferred overnight and proteins detected with primary antibodies from Santa Cruz Technologies, ABCam, Millipore and Cell Signaling Technologies followed by appropriate HRP-conjugated secondary antibodies and ECL detection reagents. All represented westerns have been reproduced at least 3 times from different experiments.

5.10 Inhibitors and stimulators

Stock of each inhibitor in DMSO or water was prepared as directed by Calibiochem or Sigma and stored in aliquots at -20°C. The following inhibitions were used:

a. p38 inhibitor: SB202190-Competitive inhibitor of p38b activity and ATF2 activation, reversible (20µM).

b. p38 inhibitor: SB239063-ATP competitive inhibitor of p38a and p38b, reversible (10µM).

c. p38 inhibitor: SB203580-ATP competitive inhibitor, reversible (20µM).

d. JNK inhibitor: (L-form)-Blocks the phosphorylation of the activation domains of JNK (20µM).

e. JNK inhibitor: SP600125- Complexes with ATP, blocks cJun transcription, reversible (10µM)

f. Stimulator JNK: Anisomycin from Streptomyces griseolus. Affects DNA and protein synthesis (0.005M).

g. Stimulator p38: Sorbitol. Sugar that influences the overall osmotic concentration in cells (0.2M).

DMSO working and control concentration was 0.5µl/ml in HBSS/spent media. DMSO concentration was maintained at 0.5% (vol/vol) in all media containing DMSO irrespective of the inhibitor concentration.

The inhibitor concentrations used were non-toxic to cells as cell morphology and cell counts to calculate percent viability (trypan blue) are similar in each cell culture treated with inhibitors or DMSO.

Cells were pretreated with their respective inhibitors for thirty minutes prior to infection and inhibitor was present during adsorption, during infection and during the replacement of media after infection (24hrs). The media left after the one hour adsorption period consists of presence or absence of the inhibitor and 2 percent FBS.

5.11 Plaque Assays

Vero cells were seeded in 12-well flat bottom plates and serial dilutions (10^{-1} to 10^{-6}) for collected lysates were run in duplicate. After 1 hour adsorption, unadsorbed virus was removed and media replaced with replacement media that comprised of a 1 percent methyl cellulose, 2 percent fetal bovine serum (FBS) mix. This was left for 48 hours, then the infection was stopped by fixation with 100 percent methanol, and monolayers stained with crystal violet or by immunofluroescense for the detection and the observation of plaques.

5.12 Viral kinetics

Cells were infected at MOI 5 as described in infections for efficiency of plating and infections. After the one hour adsorption period, media was replaced with 2% FBS media and at stated time points samples collected for DNA-analysis, RNA-analysis or total cell lysates collected separately for SDS-PAGE analysis and for plaque assay (titration of pfu/ml at the time point). B virus glycoproteins gC and gD as well as B virus protein US11 were probed for in total cell lysate collected for SDS-PAGE analysis.

5.13 FACS (flow cytometry) analysis

Cells were untreated or mock-infected or infected with B virus at MOI 5, and at defined time points, media was removed and enzymatic digestion at 37°C was used to lift the adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4% to 8%. The fixation solution was removed, and cells were centrifuged. These steps were followed by permeabilization and resuspension by the slow addition of 90% methanol. Cells were equally aliquoted and incubated in blocking buffer at room temperature for 10 minutes and then rinsed by centrifugation in incubation buffer. This was followed by primary antibody incubation, washes and fluorochrome-conjugated, secondary antibody incubation. Washes were then done and cells resuspended in 0.5 ml PBS for detection/reading in the cytometer. FACS limits were for 10000 events and data is representative of duplicate experiments. For each experiment the histograms show the untreated unstained for that time point with the stained mock infected or stained B virus infected for that time point.

5.13.1 ELISA/Luminex-Liquichip

Cells were prepared to be ready in 2 to 3 days for infection. Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume 1% FBS media. At the defined time points, post absorption supernatant was removed, spun for 5 minutes at 1000 rpm to pellet any cell debris and the addition of Tween/DOC done (i.e., add 10% Tween and vortex, followed by 10% Doc to make Tween and Doc 1% of the final concentration). After removal from BSL4, 100 ul aliquots were prepared and stored at -20°C. Prior to usage for readings, the supernatant was vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. Note that for each technique used, Luminex or ELISA a supplied negative for each cytokine was supplied and the values for each supplied negative was represented what is traditionally called the blank in ELISA. This reading received for each supplied negative (N value) was used to calculate P/N values for each treatment (mock cell lysate or B virus) ie P value, such that the charts represent the P/N values for both treatments through time. This method did not allow for masking of any observed effects due to the mock treatment to occur.

6 Results

6.1 Specific Aim 1: Establish *in vitro* target cell models representing the cells that are initially Infected during acute infection in order to study early virus:host interactions.

6.1.1 Isolation of cells

Each of the three batches of tissue received throughout the year (April, July, and August) via a tissue sharing program with Yerkes Primate Center, Emory University, Atlanta GA, led to differing cell retrieval successes via the classically established method presented in the Materials and Methods section. The differences in success were due to problems encountered with contamination, isolation of cell type, propagation of cells for passage and storage, as well as determination of appropriate media cocktail and additives necessary for long-term growth and storage.

In brief, with the first (April) batch of tissues, I had success only in isolating and seeding keratinocytes and fibroblasts. However, with time, fungal contamination became a major issue with the fibroblasts and no cells were salvaged. The contamination also made it difficult to determine which media cocktail was appropriate for long-term use and storage of the macaque fibroblasts.

With the second (July) batch of tissues, I once again had limited success in the isolation of keratinocytes and the keratinocytes survived only 45 days. I also had problems again with contamination of the fibroblasts which were the intended cells for isolation in this aim..

On receipt of the third (August) batch of tissues, I specifically worked with the skin and foreskin tissue sections only because I had isolated glial or satellite cells which were now stored in the liquid nitrogen. With the third (August) batch, I was able to successfully isolate keratinocytes and fibroblasts (foreskin) without any contamination. It should be noted after attachment, cells initially grew slowly as they established a population within the flasks or wells. The fibroblasts stored and used for this dissertation work were derived from foreskin because contamination was a constant problem with skin-derived fibroblasts. Twenty-two tubes of passage 2 rhesus macaque foreskin fibroblasts were obtained. Regarding cells seeded in the T-25 flasks, the small populations were transferred into 6-well plates, as the surface on the T-25 may have been too large for them to maintain the contact necessary for growing. The cells maintained a healthy appearance as they began grow and multiply.

Primary macaque fibroblasts were successfully isolated and passaged for use in all future experiments described.

6.1.2 Verification of no bacteria, fungal or mycoplasma contamination

Subcultured cells and their media were observed over several weeks and proved to be consistently healthy and free of contaminants. PCR analysis for mycoplasma also showed that cells were contaminant free. Additionally, fluorescent staining of nuclei indicated the absence of mycoplasma.

6.1.3 Visualization of morphological characteristics and passage stability

Morphologically the fibroblasts maintained their characteristic structure with each subsequent passage: branched cytoplasm, elliptical speckled nucleus with one or two nuclei and abundant rough endoplasmic reticulum (RER) (Figure 22).

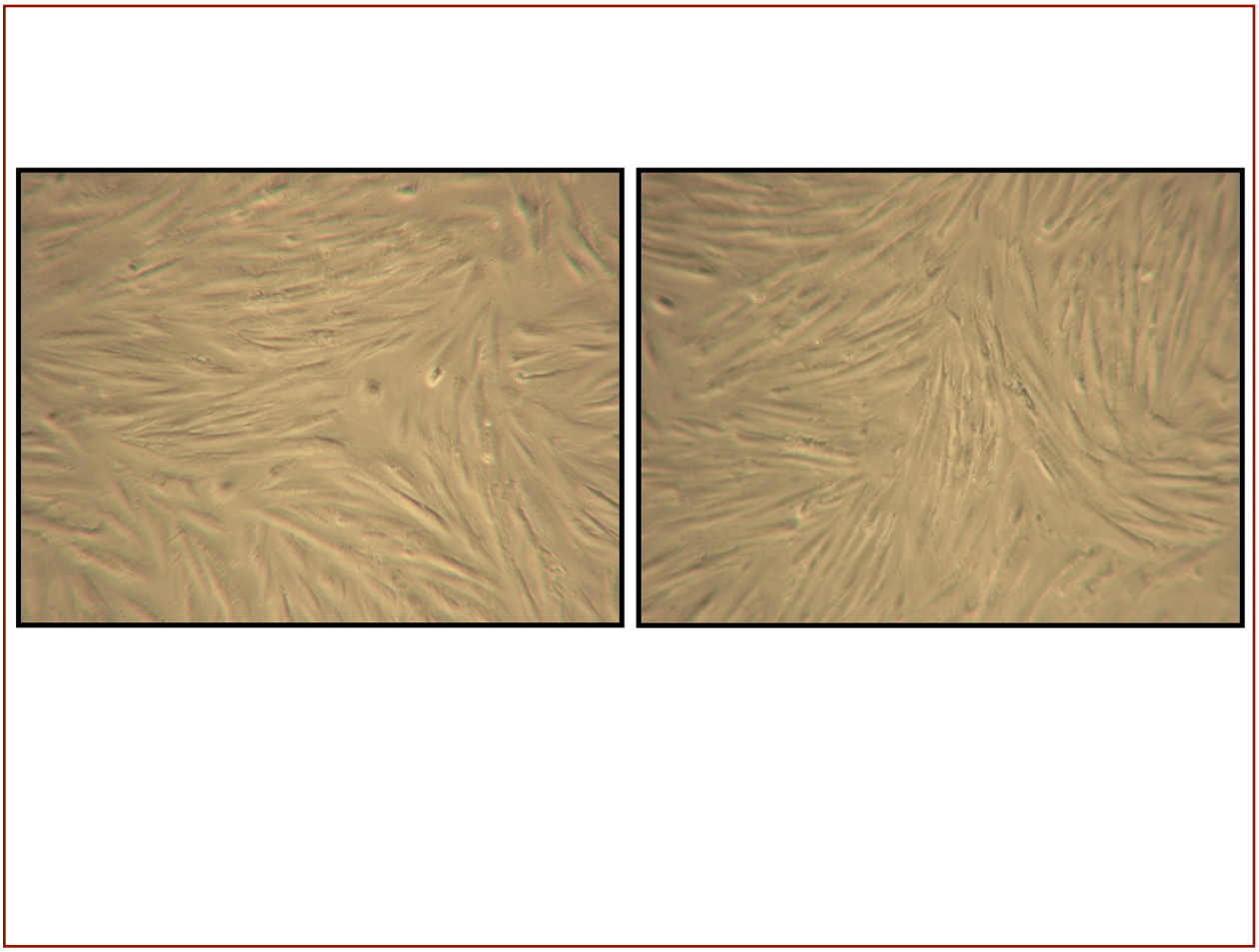


Figure 22. Rhesus macaque fibroblasts (RMF) isolated and established successfully in Specific Aim 1.

They also had the characteristic disorganized and dispersed pattern that is associated with fibroblasts in a large area of space, and gradually became more parallel in alignment as

they neared each other and/or approached confluency (Figure 22 and Figure 23). Staining for fibroblast cell markers vimentin and fibronectin and staining for actin to view cell shape/morphology also verified that these cells were fibroblasts.

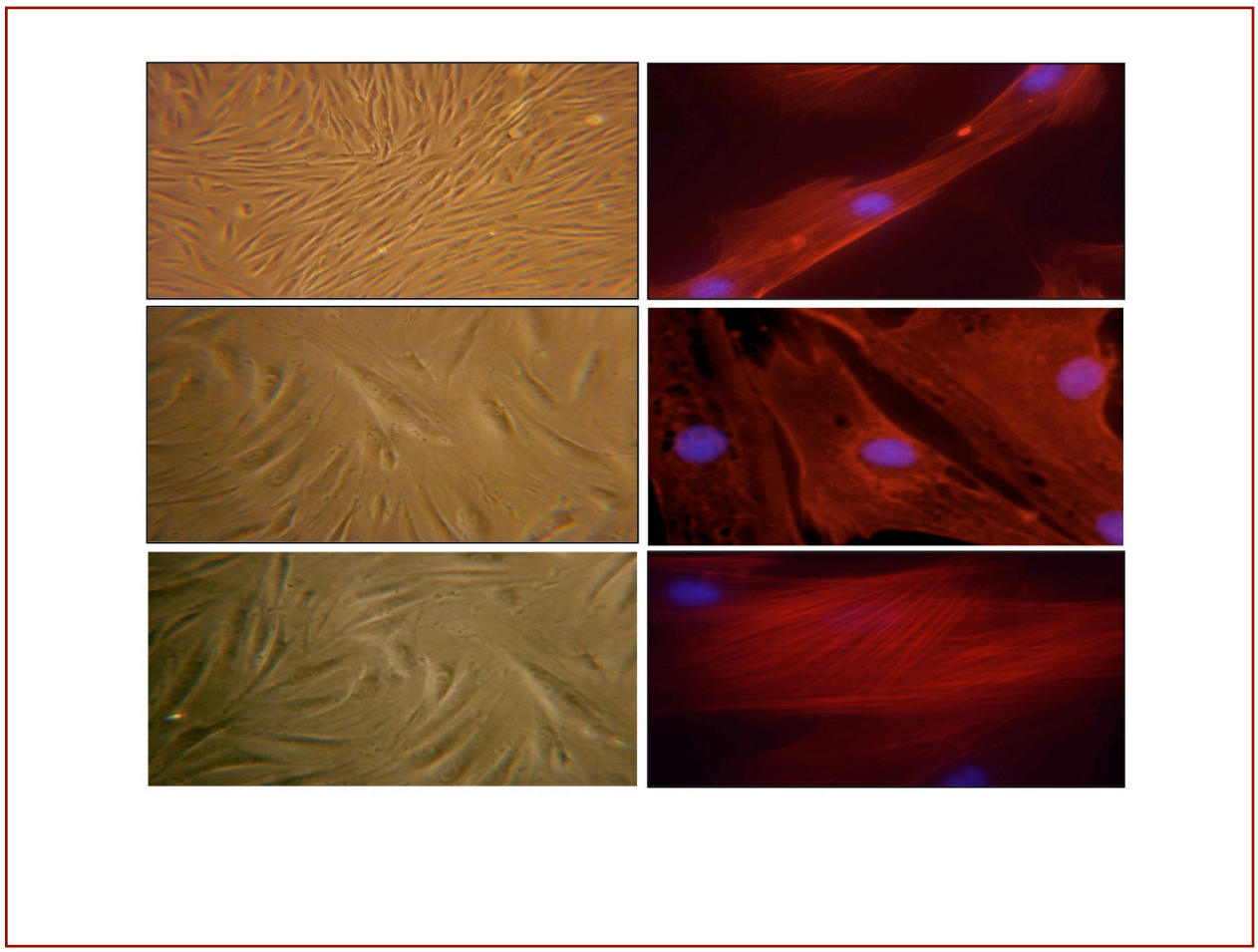


Figure 23. RMFs showing typical fibroblast characteristics

Branched cytoplasm, elliptical speckled nucleus with one or two nuclei, abundant rough endoplasmic reticulum, and parallel alignment as confluency is approached.

As with any primary cell line, the division of cells slowed with increased passaging (p12 and higher), and as confluency approached 100% senescence occurred, resulting in a slower growth rate in any subsequent passage. Morphological characteristics also changed with in-

creased passage, in that cells became more spindle-shaped and thinned, with a ragged appearance.

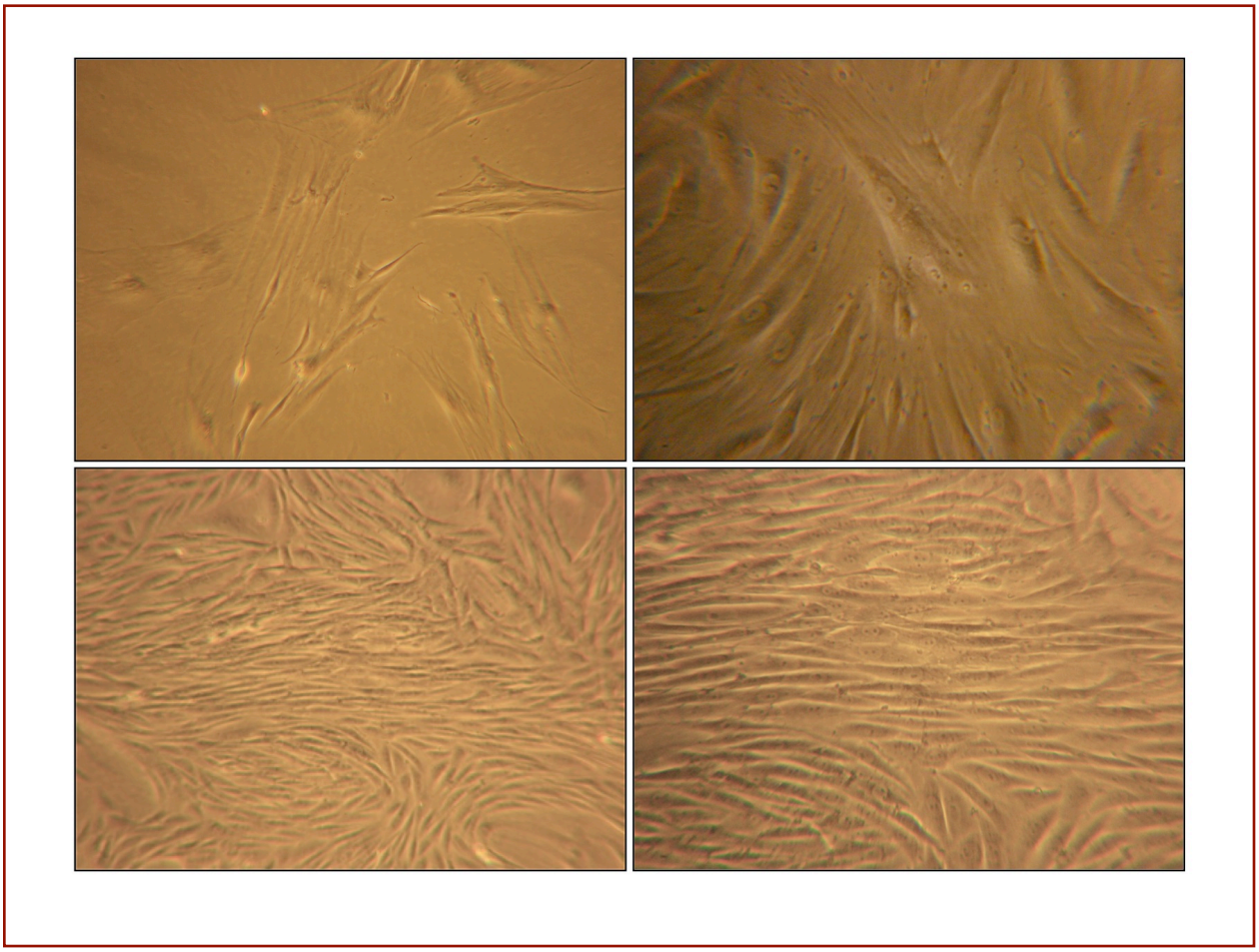


Figure 24. RMFs showing parallel alignment as confluency is approached

6.1.4 Plating efficiency

The overall purpose in determining the efficiency of plating is to equalize the multiplicity of infection (MOI) for future infections with the stated virus in the different cell types. Vero cells were used as they are a susceptible cell line that is classically used for the titration of HSV-1, HSV-2 and B virus stock as well as many other viruses. All B virus stock preparation and de-

termination of their pfu/ml for the stated stick is done in Vero cells. Therefore by comparison of the calculated pfu for B virus in the human fibroblasts and the monkey fibroblasts compared to Vero cells allows for one to calculate the fold difference in pfu/ml for each cell line compared to the pfu/ml for the Vero. With this calculation one is able to determine the fold increase or decrease in pfu/ml compared to Vero needed to equally infect the human and macaque fibroblasts at defined multiplicity of infections (MOIs).

The general fold difference compared to Vero cells is outlined below (Table 4) for the stated cell line, with the stated virus, as determined by parallel plaque assays performed. Vero cells were used, as they are the classical susceptible cell line used for titrations done (plaque assays) to determine the virus concentration in prepared B virus stock.

Table 4 Efficiency of plating fold comparisons for the stock of B virus used

	B Virus (E2490)
HFF (Human foreskin fibroblasts)	**6 fold less** **(B Virus pfu in HFF)/(B Virus pfu in Vero)**
RMF (Rhesus macaque fibroblasts)	**0.5 fold less** **(B Virus pfu in RMF)/(B Virus pfu in Vero)**

For each new stock of virus that was prepared or used, the necessary titrations followed by plaque assay on Vero cells were done to determine the efficiency of plating for that stock of virus (as shown in table above). In order to determine the amount of virus needed (plaque forming unit (pfu)) to equally infect each cell line at a defined multiplicity of infection (MOI) the difference in pfu for each cell line was compared to the calculated pfu for the Vero cell titration (plaque assay) with B virus. Figure 25 and Figure 26 show the general plaque formation after infection of RMF cells with B virus.

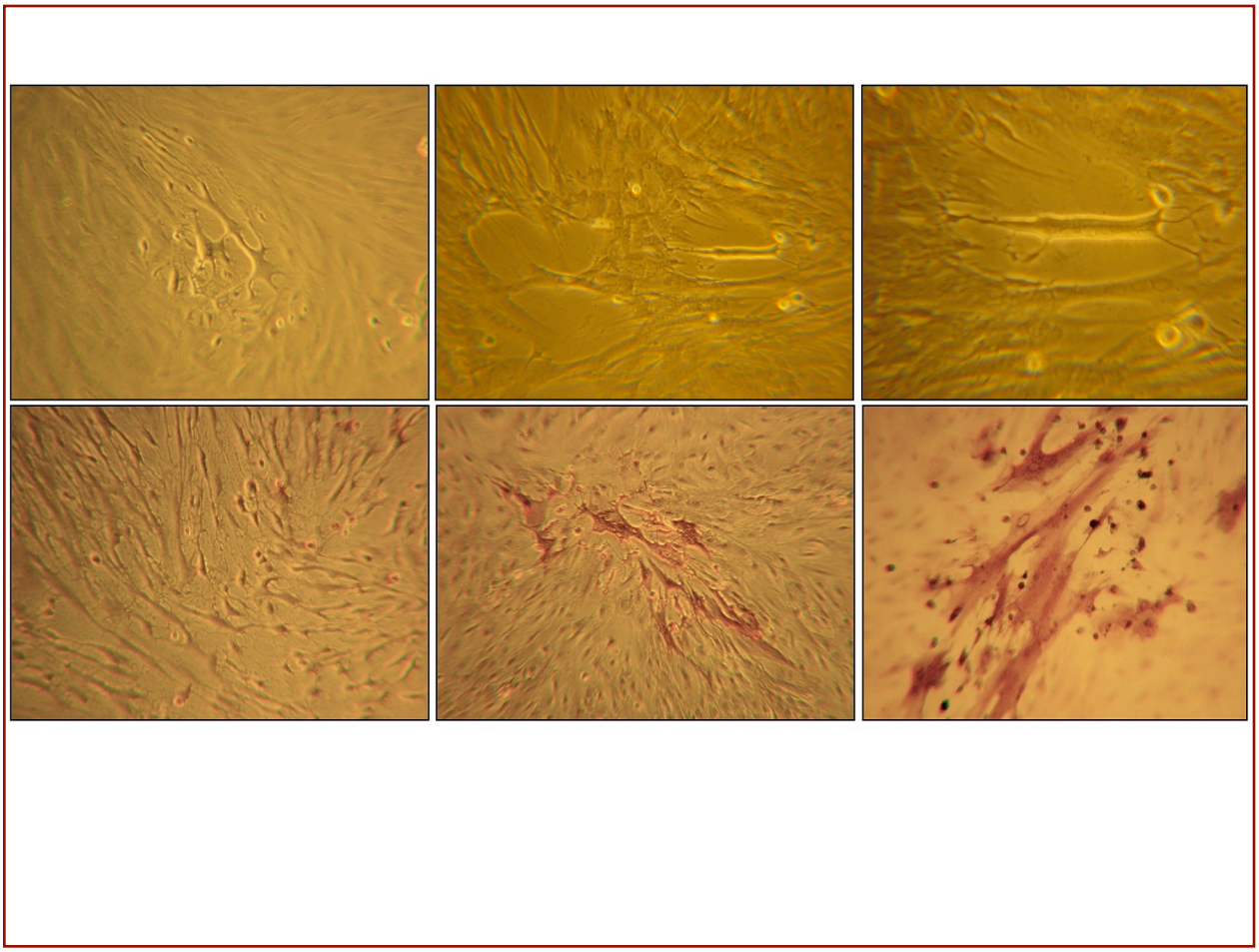


Figure 25 Plaque formation after infection of established rhesus macaque fibroblasts with B virus

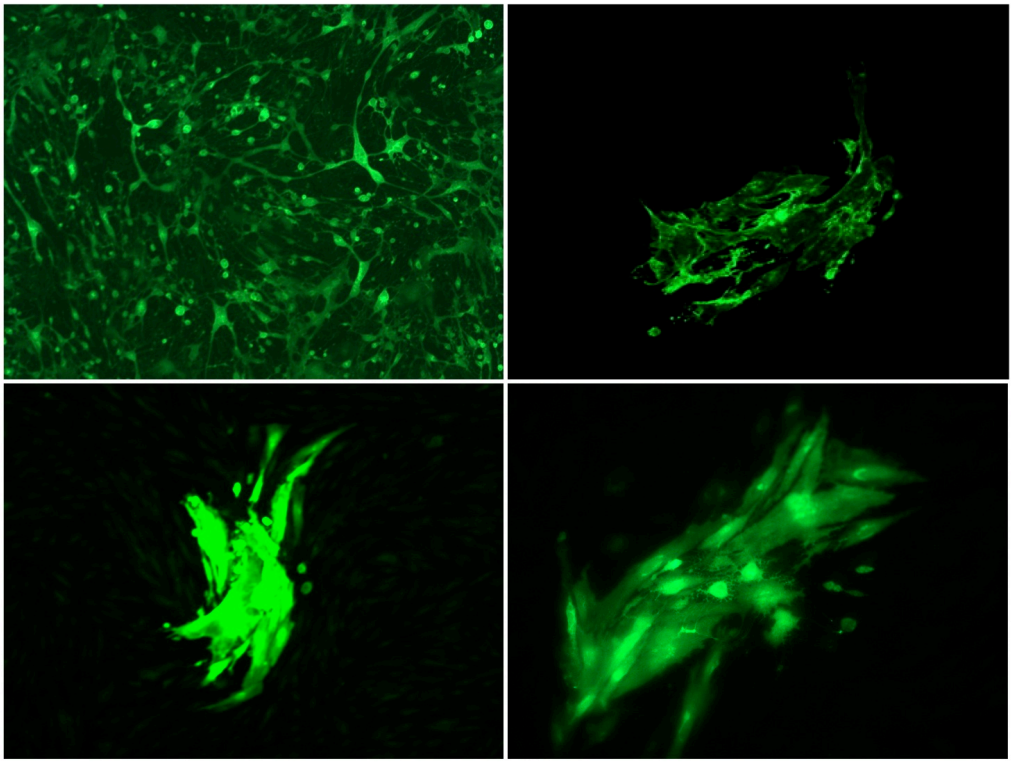


Figure 26. Plaque morphology observed after infection of established rhesus macaque fibroblasts with B virus.

6.1.5 Kinetics of B virus replication in HFF, RMF and Vero cells

The kinetic analysis of B virus in each of the three cell lines is shown in Figures 27 and 28 depicting virus quantification and viral protein production, respectively. The goal of this analysis was to establish the ability of B virus to replicate in the three cell lines and assess the expression of viral glycoproteins during replication. Over a twenty-four hour period we observed that the amounts of viral DNA and the pfu/ml increased through time. We also observed that there was expression of viral glycoproteins (gC and gD) and that the expression of viral protein

US11 increased through time in all three cell lines establishing the competency of the cells to replicate B virus.

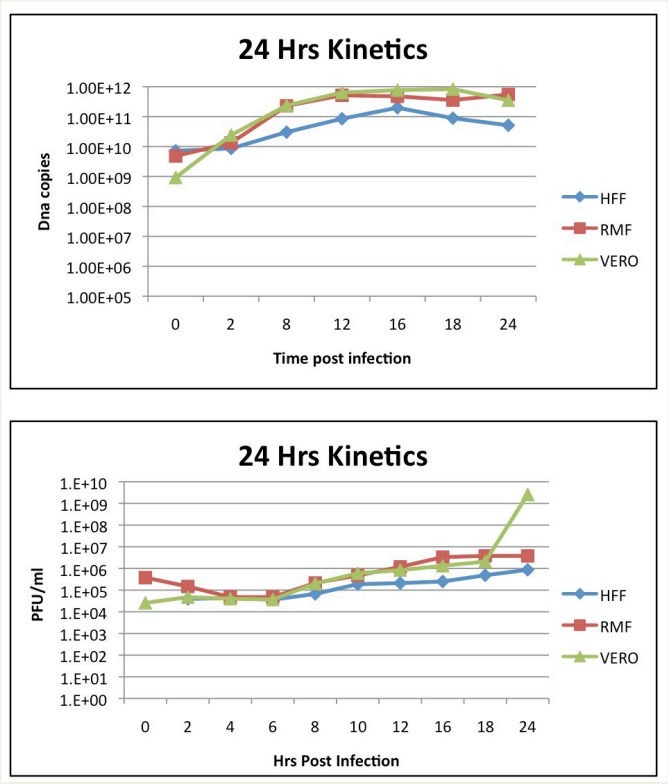


Figure 27 Kinetics of B virus infection in three different cell lines

The kinetics of B virus infection is shown for human foreskin fibroblasts (HFF), Rhesus macaque fibroblasts (RMF) and African green monkey kidney cells (Veros) over a 24 hour period. The top chart represents DNA copy number and the bottom represents plaque-forming units/ml (pfu/ml). Human (HFF) and monkey (RMF) cells were infected with B virus (BV) MOI 5 and at defined time points samples were collected for DNA analysis by PCR or total lysate collected for plaque assay. Data over this 24hour period were collected only once for kinetic analysis but each point was evaluated from replicate wells that were averaged for this figure. PCR analysis reflects total particle number, whereas PFU/ml reflects replication competent virus. A 1:10,000 particle-to-PFU ratio, as seen from DNA copy number/PFU ratio, is commonly reported for animal viruses.

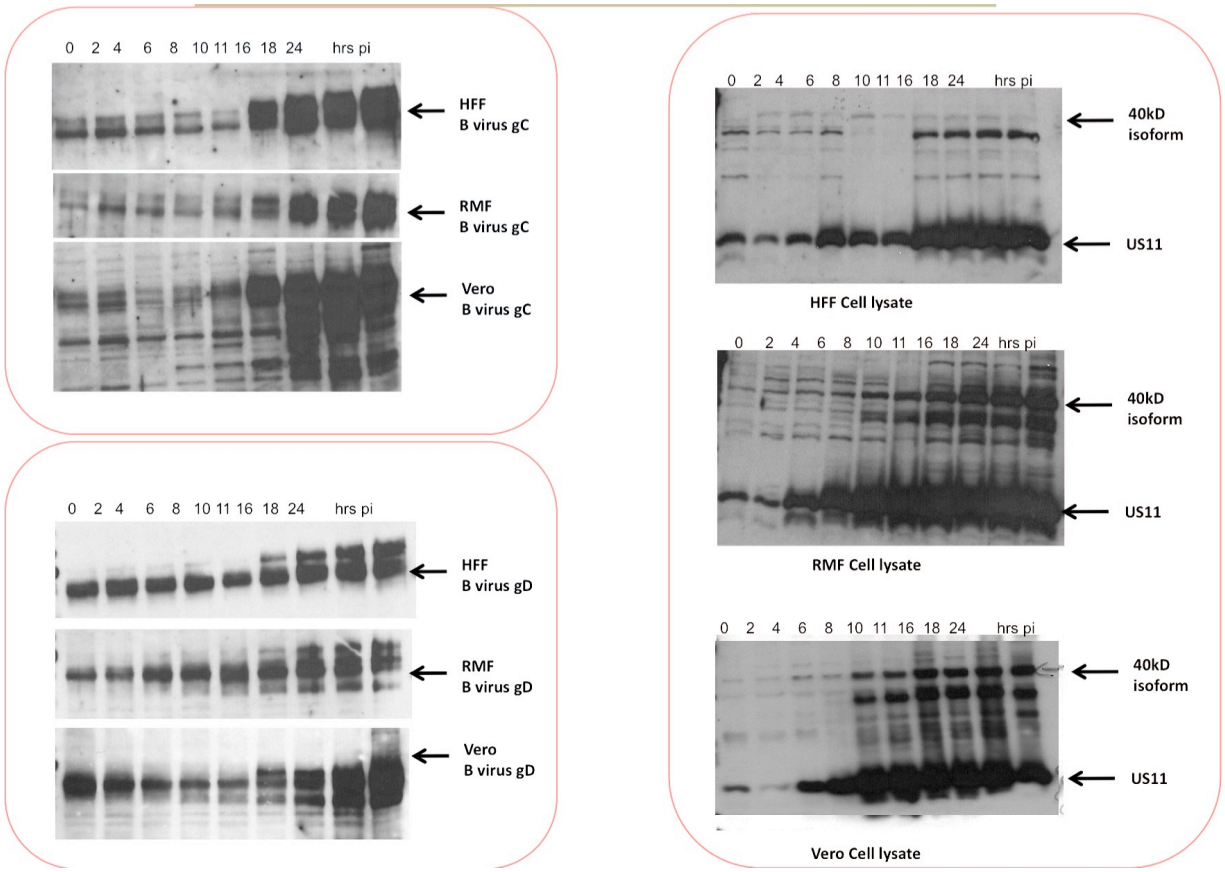


Figure 28. Viral protein production at different time points post B virus infection in each of three cell lines.

Representative western blot analyses for late protein synthesis, glycoproteins gD and gC, as well as viral protein US11 levels during 24 hours of infection with B virus in HFF, RMF and Vero cells are shown in each panel. Human (HFF) and monkey (RMF) cells, as well as Vero cells were infected with B virus at MOI 5. At defined time points total cell lysate were collected from each cell line using SDS lysis buffer with added HALT phosphatase inhibitors. SDS- PAGE and Western blotting detection procedures were carried followed by ECL immunodetection.

6.2 Specific Aim 2: Identify the specific MAPK responses induced by B virus in acutely infected cells.

6.2.1 Adaptor proteins recruited after B virus infection

To determine which signaling pathways are induced by B virus infection of macaque and human fibroblasts we initially investigated the contribution of three adaptor proteins (six adaptor proteins total) known to play a role in relaying pathogen associated molecular patterns received by pattern recognition receptors to associated signaling pathways, because these (adaptor proteins) serve as points for signals to be integrated and relayed such that cellular signaling pathways, such as the MAPK pathway, is activated. The results of these experiments are seen in Figure 29.

We measured the levels of phosphorylated TRAF2, TRAF6 and TRIF proteins after B virus infection and found comparable to to cells exposed to uninfected cell lysate controls. Levels of TRAF2at 30 minutes post infection in the macaque cells are relatively larger than that observed for the human cells. The larger band observed is likely due to activation of TRAF2 recruited to adaptor complex formation following exposure of macaque fibroblasts to both uninfected and infected cell lysates. Curiously, no indication of virus specific TRAF2 or TRAF6 activation was noted in spite of the observation that MAPK pathways were engaged following exposure of both macaque and human cells to B virus. Each of these adaptor molecules are common to the MAPK pathway.

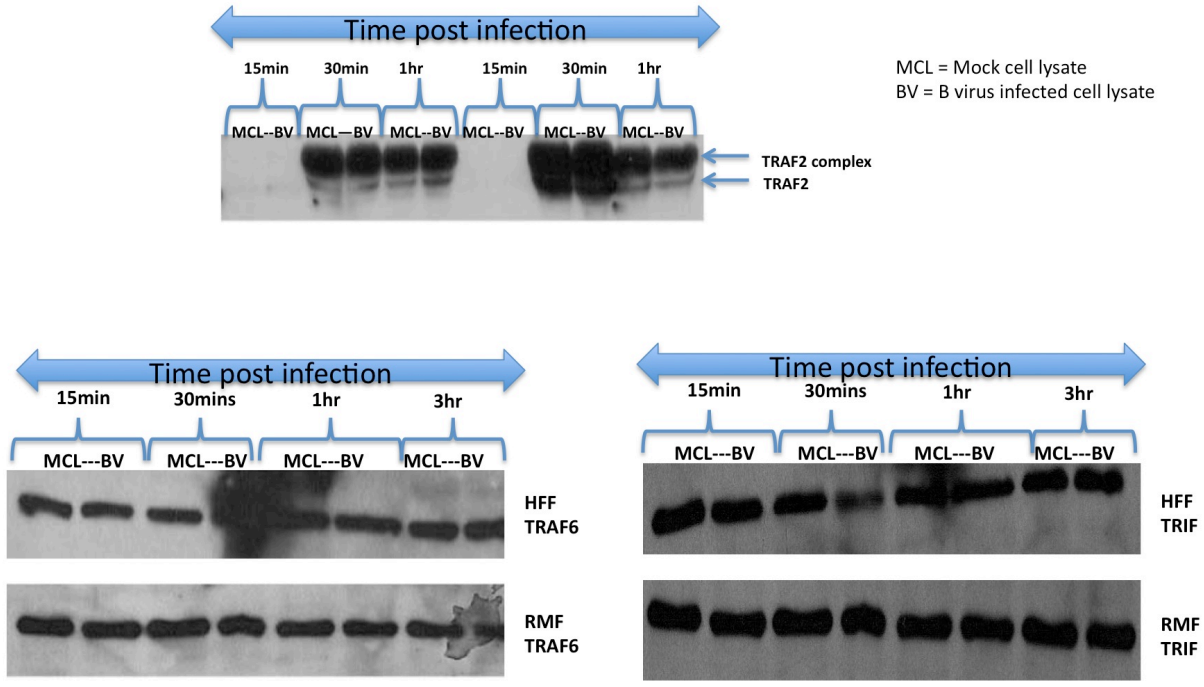


Figure 29. Adaptor proteins recruited after B virus infection
Human (HFF) and monkey (RMF) cells were either infected with B virus (BV) or exposed to mock infected (MCL) cell lysates at MOI 5. At defined time points cells were harvested in SDS lysis buffer with added HALT phosphatase inhibitors. SDS- PAGE and Western blotting detection procedures were carried followed by ECL immunodetection.

6.2.2 Verification of JNK and p38 activation after B virus infection

The points of convergence from the adaptor proteins allow for initial signals from the PRRs to be relayed to the MAPK pathway. I therefore sought to verify that the MAPK pathway was activated after B virus infection.

Analysis of western blots revealed that JNK1 and JNK2 were activated by 1-5 hours post infection in both cell lines in a non specific manner, .i.e., exposure of cells from both species to either infected or uninfected cells resulted in activation (Figure 30). JNK2 activation lagged until 3 hours post infection in macaque cells, but not in human cells, which would potentially result in delays in the downstream events of this activation process. By five hours post infection JNK2 activation was almost complete inapparent and data suggested a monophasic increase at three hours unlike the pattern of activation in humans cells. JNK 3 isoform was activated slightly in a virus specific manner across all time periods in human cells, but again showed a monophasic pattern of activation at three hours post infection in macaque cells, followed by a nearly completely loss activation at five hours. This raises the question of the importance of each of the isomeric forms of JNK, an area which is largely unexplored. Though JNK3 is thought to be expressed mainly in heart, brain and testis tissues and play a role in the stimulation of apoptosis, our data show that fibroblast cells do express JNK3 and B virus effectively shuts down JNK3 activation in a biphasic manner at one hour and five hours post infection specifically in human cells (Figure 30) and completely in macaque cells. The role each of these isoforms play in downstream events has not been reported in any system to date. FACS data of each time point largely support western blot data, establishing that there are virus-specific modulations of JNK activation, with clear differences in human versus macaque cells with the exception of three hours post infection in human cells. No isoform studies could be performed with FACS since antibodies for this technique were not commercially available.

Activation of p38 was observed to occur in a virus-specific manner at one hour through three hours post infection in the foreign host cells (HFF), whereas in the natural host cells (RMF)

there was a relative increase in virus-induced phosphorylation of p38 (pp38) only at three hours post infection (Figure 31). This monophasic activation pattern is similar to observations made regarding JNK activation. Interestingly, macaque cells may be more sensitive at detecting and repsonding to perturbations caused by exposing the cells to uninfected as well as infected cell lysates as can be seen on western blot analysis. Human cells exposed to uninfected cell lysates did not activate p38 until five hours post infection.

MAPK phosphatase (MKP) is known to play a role by dephosphorylation to regulate the MAPK responses influenced by pJNK and pp38. Our data showed that the activity of MAPK phosphatase 1 (MKP1) decreases in both hosts at three hours post infection through five hours post infection (Figure 32) in a virus specific manner, whereas cells treated with uninfected lysate continue to show realtively abundant levels of MAPK phosphatase. This decrease in phosphatase activity may explain the increaed levels of activated p38 and those JNK isoforms that are activated, but it is clear that B virus is specifically regulating activation and perhaps de-activation. Interestingly, phosphatase activities appear to be similarly regulated in cells of both specieis in both virus dependent and independent manners.

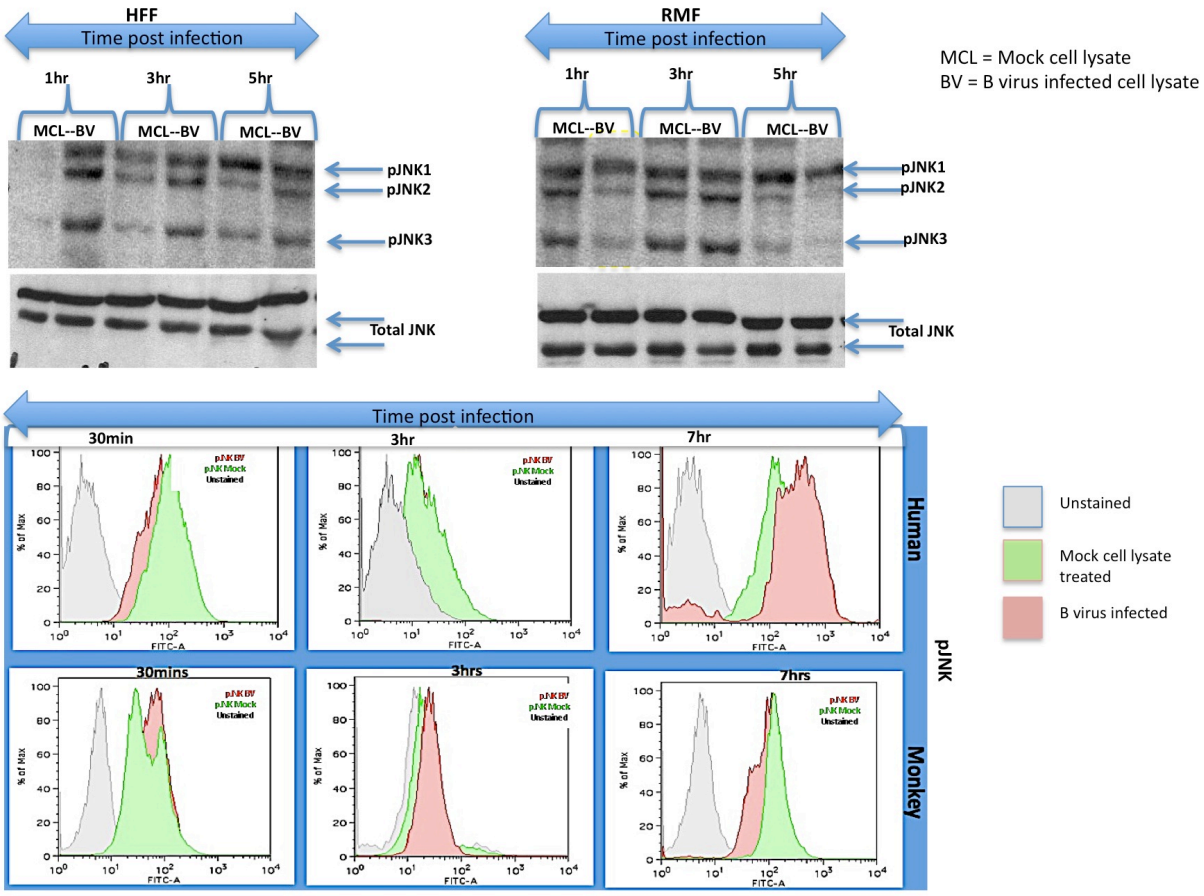


Figure 30. Activation (phosphorylation) of JNK after B virus infection

Human (HFF) and monkey (RMF) cells were either infected with B virus (BV) or mock infected (MCL) at MOI 5. At defined time points total cell lysate (a) for western blotting procedures were collected from each cell line with SDS lysis buffer with added HALT phosphatase inhibitors. SDS-PAGE and Western blotting detection procedures were carried followed by ECL immunodetection, (b) for FACS analysis all cells were removed by enzymatic digestion. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fix was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. FACS limits were for 10000 events and data is representative of duplicate experiments and staining with defined primary and secondary antibodies for analysis by flow cytometry.

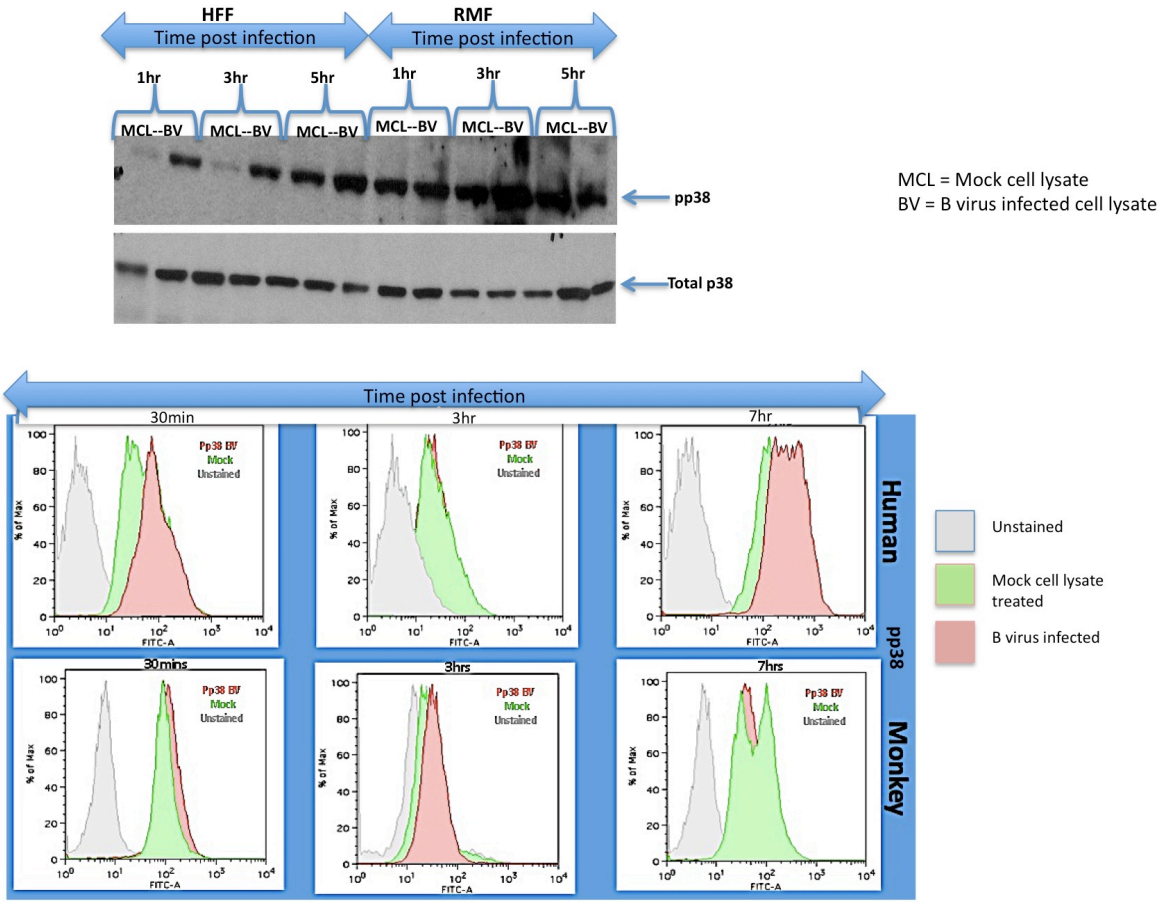


Figure 31. Activation (phosphorylation) of p38 after B virus infection

Human (HFF) and monkey (RMF) cells were either infected with B virus (BV) or mock infected (MCL) at MOI 5. At defined time points total cell lysate (a) for western blotting procedures were collected from each cell line with SDS lysis buffer with added HALT phosphatase inhibitors. SDS-PAGE and Western blotting detection procedures were carried followed by ECL immunodetection, (b) for FACS analysis all cells were removed by enzymatic digestion. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fix was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. FACS limits were for 10000 events and data is representative of duplicate experimentsand staining with defined primary and secondary antibodies for analysis by flow cytometry.

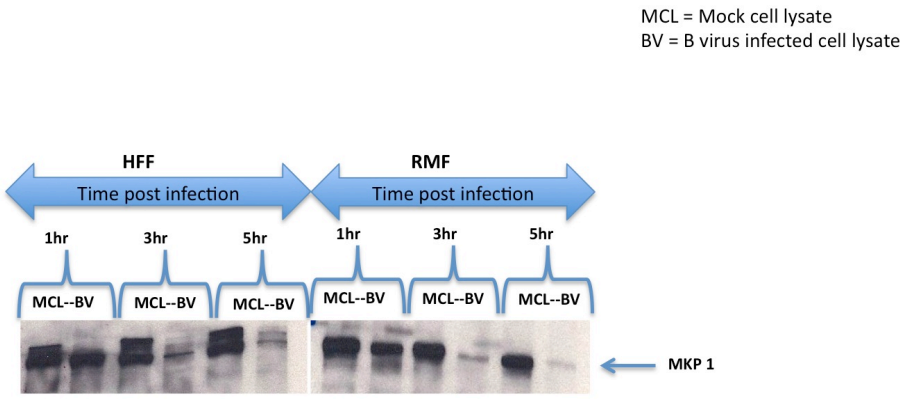


Figure 32. MAPK phosphatase (MKP) levels after B virus infection

Human (HFF) and monkey (RMF) cells were either infected with B virus (BV) or mock infected (MCL) at MOI 5. At defined time points total cell lysate for western blotting procedures were collected from each cell line with SDS lysis buffer with added HALT phosphatase inhibitors. SDS-PAGE and Western blotting detection procedures were carried followed by ECL immunodetection.

6.2.3 MAP3K-ASK1 and MAP3K-TAK1 activation after B virus infection

Verification that the MAPKs p38 and JNK were differentially activated in host specific and virus specific manners underscored the question of whether MAPK-associated responses (MAPK apoptotic response) were regulated in a host-dependent manner. Therefore, the first question I asked was "Do upstream MAP3K-ASK1 (a known MAPK-associated apoptotic marker) and TAK1 become activated after infection and how is ASK1 regulatory protein 14-3-3 affected after B virus infection?" These are critical questions that when answered will ultimately drive experiments to understand host and virus specific mechanisms of regulating disease outcome that so distinctly separates these hosts during B virus infection.

Western blots were performed using cells collected at different time points post infection in both human and macaque cells and probed with antibodies specifically reactive with activated TAK1, ASK1, and 14-3-3. Data from replicate experiments shows that pTAK1 (Thr184/187) dramatically decreases through one hour to five hours post infection in human cells relative to those cells treated with uninfected cell lysates, whereas it dramatically increases in macaque cells. In the human cells, pTAK1 (Ser412) had decreased protein levels at 5 hours post infection whereas after the same amount of time, protein levels had increased in the macaque cells (Figure 33). These are striking differences in the way which human and macaque cells respond to B virus infection. In the absence of TAK1 activation, human cells infected with B virus will fail to mount a proinflammatory response and lacking this response, and virus replication can proceed unhampered by host defense systems, allowing the virus to spread at high levels through the sensory ganglia to the CNS. With respect to ASK1, if activation

occurs cells can activate p53 and apoptotic defenses can take place. Interestingly, ASK1 is activated immediately after infection in human cells (1 hpi), and slightly later in macaque cells (3hpi), but levels of activation are low and limited to these single time points, suggesting that cells from both species can respond to B virus infection, but only for a very short interval and weakly with respect to engaging in apoptosis defenses. The mechanism by which B virus suppresses ASK1 activation will be the topic of future studies. Limited activation of ASK1 will also result in limited intervals of MAP2K activation leading to dampening JNK and p38 activation.

The regulation of MAPK-dependent apoptosis can occur through the MAP3Ks to signal pro-apoptotically via a MAPK (p38, JNK), to influence inhibitory proteins such as the 14-3-3 or to influence activation proteins such as p53 (p53 has been found to complex with JNK for its sequestration and subsequent degradation). Our data show that in B virus infected human cells the presence of activated ASK1 (pASK1) can be observed at only one hour post infection (Figure 33) and only weakly at three hours post infection in macaque cells. As previously mentioned, ASK-1 is a MAPK3K that can be regulated by the family of 14-3-3 proteins. Is 14-3-3 responsible for the short, relatively weak activation of ASK-1? This family of proteins have been shown to diffusely spread within the cytoplasm of cells and be regulated by many factors such a p53, which is also regulated by ASK1. The 14-3-3- proteins are considered to be regulators of the cell and the cell cycle as they allow for: activation of MAPK, prevention of apoptosis, cells to arrest in G2 phase and bind to mitosis specific translation/initiation factors such as EIF4B, suppression of cap-dependent translation and stimulation of cap-independent translation (thus imparing mitotic exit). Therefore we examined by western blotting, the relative levels of 14-3-3 proteins

after B virus infection. We observed that there were no differences in the relative levels of 14-3-3 in any cells whether they were exposed to infected or uninfected cell lysates. (Figure 33).

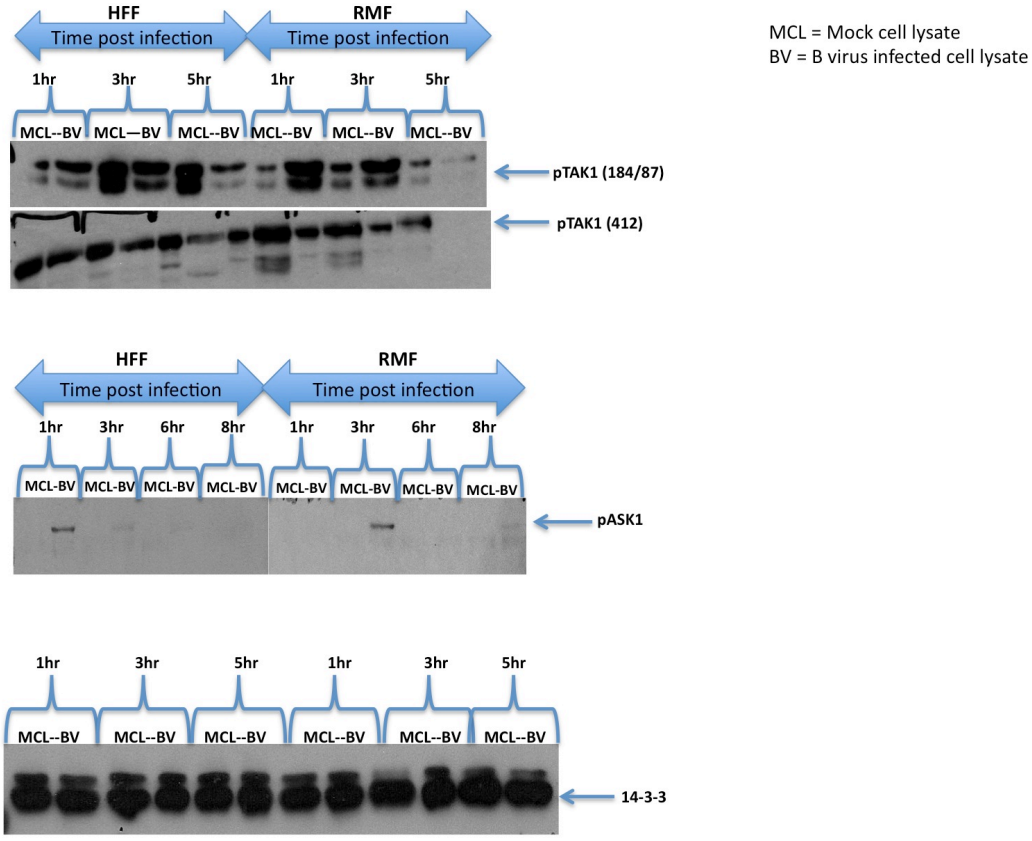


Figure 33. MAP3K (ASK1 and TAK1) activation and regulation by 14-3-3 proteins

Human (HFF) and monkey (RMF) cells were either infected with B virus (BV) or mock infected (MCL) at MOI 5. At defined time points total cell lysate for western blotting procedures were collected from each cell line with SDS lysis buffer with added HALT phosphatase inhibitors. SDS-PAGE and Western blotting detection procedures were carried followed by ECL immunodetection.

6.2.4 Induction of apoptotic events pp53 and cleaved caspase 3 after B virus infection

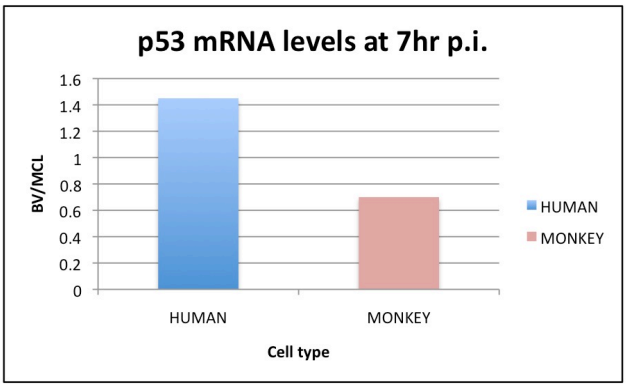
From the data presented thus far, we know that a productive infection is established in cells from each species, as seen in the kinetic anaysis of virus replication, albeit at with reduced effiency in macaque cells. Thus, there is the potential for spread to neighboring cells however, with the observed, different host outcomes (survival vs. death). We also know from the work of a number of investigators that that herpesviruses (such as HSV1) are known to inhibit the host apoptotic response within the initial three to five hours post infection. With this knowledge, we next investigated whether downstream apoptotic events were influenced by MAPKs (pp53 and cleaved caspase 3) after B virus infection.

Cells from each species were infected with B virus and harvested at 7 hpi. This timepoint was selected to allow for the effects of events at 5 hpi to unfold. Cells were harvested and p53 mRNA was measured in cells from each species with levels expressed as a ratio of infected to uninfected cells. Replicate cultures were pooled for this analysis. The levels of p53 were two-fold greater in human cells when compared to macaque cells, however differences in the patterns of p53 activation (pp53) were apparent in cells from each species as seen in Figure 34. Human cells infected with B virus do not show increased p53 activation until 5 hpi, whereas by this timepoint macaque cells have almost no serine phosphorylation of p53 while maintaining serine 46 phosphorylation p53 in cells exposed to either infected or uninfected lysates. Thus, serine 46 appears to be phoshorylated regardless of the presence or absence of B virus in macaque cells, whereas in human cells serine 46 is phosphorylated in what appears to be a B virus specific manner. The implications of this differential phosphorylation are unclear, however, it is remarkable that macaque cells appear to respond via p53 activation when exposed to

either B virus infected or uninfected cell lysate, suggesting that there are sensor differences between human and macaque cells. The observation that B virus infected macaque cells do not express high p53 levels of serine-15 phosphorylation by 5 hpi, whereas human cells infected by B virus show elevated levels begs further study. Interestingly, p53 (ser15) may play a role in epithelial cell responses to damage

To further investigate the apoptotic differences between B virus infected macaque and human cells, with the knowledge that caspases play a role in viral protein processing, experiments were designed to examine executioner protein caspase 3, which is cleaved when active and is responsible for the proteolytic cleavage of many apoptotic proteins. Does virus infection impact caspase 3 cleavage in a species-specific manner? Human and macaque cells were each infected with B virus at MOI 5 and harvested at 30 minutes, three hours and seven hours post infection for FACS analysis to measure activated p53 and cleaved caspase levels. The levels of cleaved or activated caspase 3 in B virus infected cells compared to the relative levels of cleaved caspase 3 in mock infected cells increase from one to seven hours post in the foreign host (HFF) (Figure 35). However, in the natural host (RMF), relative protein levels of cleaved caspase 3 never exceed that of the macaque cells exposed to uninfected cells and at three hours post infection relative protein levels of cleaved caspase 3 decrease to below those seen in cells exposed in uninfected cell lysates (control). Caspase 3 cleavage is generally considered one marker that apoptosis is in progress. Antibodies specific for activated caspase however were not sufficiently sensitive for use in western blot (data not shown). Data from the accompanying FACS analysis of activated p53 support the conclusions drawn from data shown in Fig-

ure 34. The increases in activated caspase 3 in human cells infeted with B virus correlate well with the increased p53 transcription and activation (both Ser15 and Ser46).



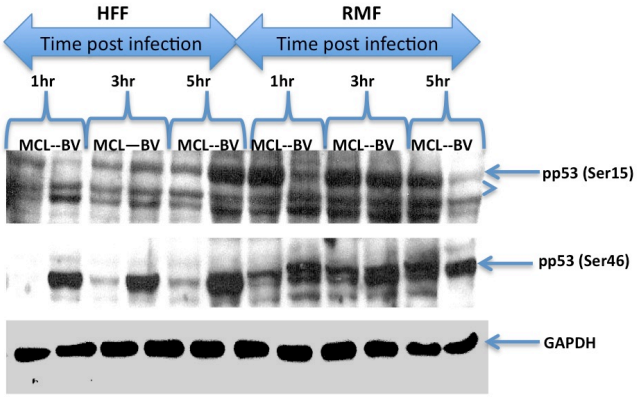


Figure 34. Analysis of markers of apoptosis p53 and cleaved caspase 3
Human (HFF) and monkey (RMF) cells were either infected with B virus (BV) or mock infected (MCL) at MOI 5. At defined time points total cell lysate for (a) western blotting procedures were collected from each cell line with SDS lysis buffer with added HALT phosphatase inhibitors. SDS-PAGE and Western blotting detection procedures were carried followed by ECL immunodetection, (b) RT-PCR was collected with RLT buffer. Total RNA was isolated and purified, followed by cDNA conversion and PCR analysis.

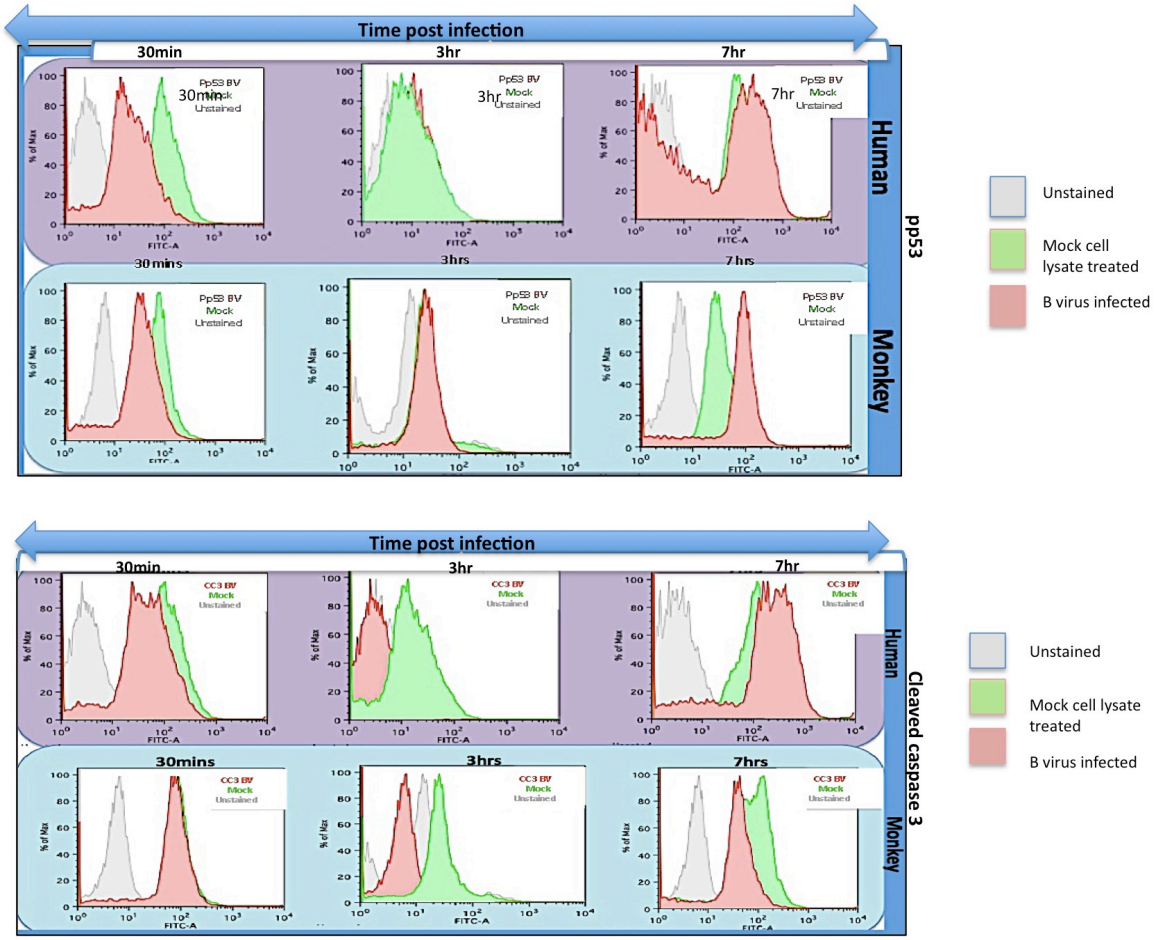


Figure 35. FACS analysis of apoptotic events pp53 and cleaved caspase 3

Cells were mock-infected or infected with B virus at MOI 5, and, at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fix was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. FACS limits were for 10000 events and data is representative of duplicate experiments**.**

6.2.5 Role of MAPKs p38 and JNK on the replication of B virus

MAPK are key drivers of the innate immune response to infection With the observed results from the analyses covered at this point, I next investigated the role of p38 and JNK on the replication of B virus by inhibition or stimulation of the pathways prior to and after infection. What are the functional consequences of the differences noted in the previously described experiments? My hypothesis was that virus replication was modulated differentially in cells of each host, resulting in differences in the amount of replicated virus able to enter the axons of the peripheral nerves. Failure to control virus load may underlie the differences in B virus pathogenesis in macaques and humans. To test this hypothesis, we infected cells of each species and evaluated the effects of blocking or enhancing the progression of the MAPK responses via JNK and p38 activation. We selected multiple inhibitors of either p38 or JNK as each group of inhibitors had different mechanisms of action.

Using each p38 inhibitor, we observed that inhibition lead to a relative decrease in B virus replication (pfu) in human fibroblasts, whereas there is was an observed increase in viral replication in the macaque fibroblasts (Figure 36). It is curious that p38 in cells from two species, albeit closely related species, have such disparate effects on virus replication. We repeated this experiment multiple times to establish confidence in these observations The essential nature of p38 in human fibroblasts for B virus replication suggests that downstream effects of this pathway are critical to some specific components of virus replication and/or assembly. , whereas in macaque cells, p38 actually may dampen the amount of B virus that is replicated and/or assembled, thus serving as a control or regulator of the amount of virus produced.

As with p38 inhibition, JNK inhibition led to a relative decrease in viral replication (less pfu) in human cell line, whereas viral replication was relatively increased in the macaque cells depending on the JNK inhibitor used (Figure 36). Manufacturers of each inhibitor supplied information with respect to the mechanism of action of each, and because of differing mechanisms of action, we anticipated potential differences in experimental outcomes of treatment, which in fact we observed. The differences in inhibitor effects, however, did not correlate with the manufacturers' descriptions of mechanisms of actions, but emerging data suggests that mechanisms of actions of these compounds may not be fully elucidated at present. Because of the curious and disparate effects in human and macaque cells, we decided to confirm these observations using a different approach, i.e., treatment of cells with p38 and JNK stimulators.

Activation of the SAPK (JNK and p38) has generally been accomplished with the use of sorbitol and anisomycin, respectively. Sorbital is a sugar and induces an osmotic shift that is stressful to cells, whereas anisomycin is an antibiotic that binds to the 60S ribosomal sub-units and prevents protein synthesis by inhibiting the peptide bond formation. Though each reagent has been used to target either JNK or p38, each induces a stress response in cells that influenced/activated the JNK and p38 pathways.

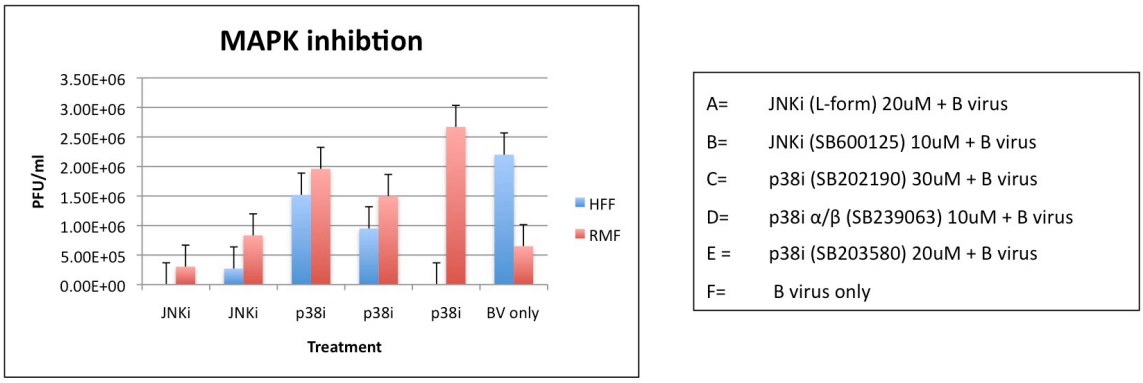


Figure 36. B virus infection in the presence of MAPK (JNK, p38) inhibition

Human (HFF) and monkey (RMF) cells were either pretreated and infected with B virus (BV) at an MOI 5 in the presence of the stated inhibitor at the shown concentration, or infected with B BV only. At 24 hours post infection total cell lysate was collected, and freeze thawed. Samples were then titrated in duplicate on Vero cells (plaque assay) and pfu/ml determined. Results are the mean ± standard error of the samples.

After stimulation of the MAPK pathways by the use of anisomycin and sorbitol, we see relative increases in the levels of pJNK3 in the human cells and relative decreases in the levels of JNK1/2/3 in the macaque cell line (Figure 37), unlike what we predicted, i.e., cells from each species would respond similarly to asnisomycin and sorbital treatment. There is no data in the literature than macaque cells may differentially utilize JNK. This observation will be explored with additional studies in the near future.

Relative pp38 levels increased to lesser extent in human cells when compared to the un-treated controls. Interestingly, the relative protein levels of pp38 are increased in sorbitol treated macaque cells when compared to controls and anisomycin treated cells. There were no observed differences in relative pp53 levels after anisomycin treatment in human cells, whereas there was an observed relative decrease in pp53 in macaque cells (Figure 37). Consistent with the effects of JNK on B virus replication in human cells, an increase in B virus was seen when JNK levels were increased as a result of anisomycin treatment. Likewise, B virus levels de-creased. These observations provided support for the results observed in the inhibition ex-periments.

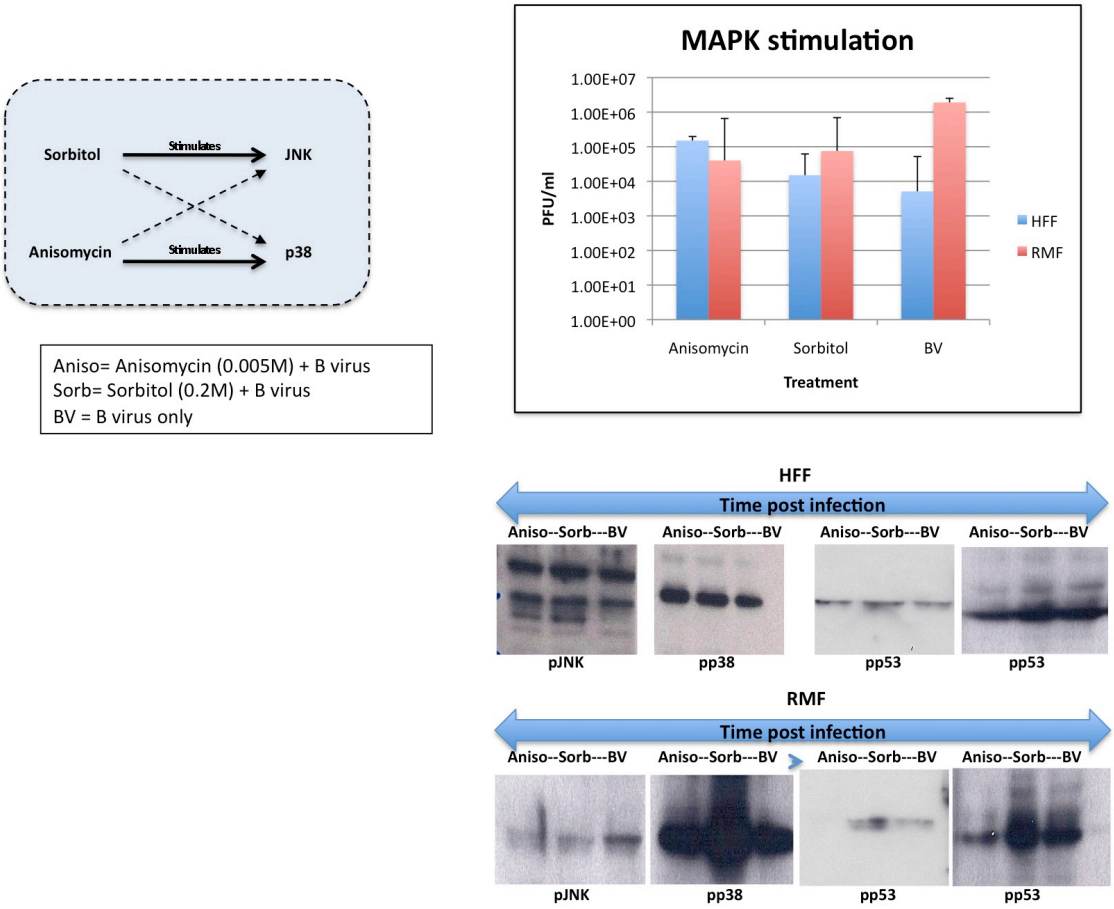


Figure 37. B virus infection in the presence of MAPK (JNK, p38) stimulation

Human (HFF) and monkey (RMF) cells were either pretreated with stated stimulator and infected with B virus (BV) at an MOI 5 in the presence of the stated stimulator at the shown concentration, or infected with BV only. At 24 hours post infection total cell lysate was collected, and freeze-thawed. Samples were then titrated in duplicate on Vero cells (plaque assay) and pfu/ml determined. Results are the mean ± standard error of the samples. Anti-phospho antibodies were used as shown with anti-pp53 antibodies specific for Ser15 and Ser46, respectively.

6.3 Specific Aim 3: Measure MAPK-induced gene products and proinflammatory mediators that are upregulated in response to MAPK-adaptor molecule activation to establish whether these events correlate with observed adaptive responses characteristic of each host species defenses against B virus infection.

6.3.1 Transcription factors associated with JNK and p38 signaling

The MAPK-JNK can influence many downstream transcription factors involved in the innate immune response to infection such as the Jun, ATF and Fos family members. Members of these families dimerize to form the AP1 transcription factor for the expression and regulation of several stress responsive genes. AP1 has been shown to be upregulated after infection with many DNA viruses and some viruses have also been shown to exploit AP1 to enhance the transcription of their viral genes. The true test of whether the differences observed in human vs. macaque cells impact the deployment of specific defenses is challenging to test in the absence of a suitable animal model that mimics zoonotic B virus infection. To overcome this obstacle, we chose to ask this question using the cell model systems representing cells derived from humans and macaques. Experiments were designed to evaluate transcription factor levels and by measuring the gene products that these factors upregulate via FACS analysis.

Human and macaque cells were each infected with B virus or treated with uninfected cell lysate as a negative control. Cells were then harvested at one and five hpi by removal of media followed by trypsin treatment to remove cells that were then treated with paraformaldehyde fixative and methanol to permeabilize cells for FACS analysis to investigate the relative transcription factor levels after infection. Histograms show that after B virus infection there was a virus-

specific shift in the activated cJun in human cell lines by five hpi times post infection (Figures 38) indicating that cJun transcription factor levels increased dramatically between one and five hours post in B virus infected human cells (Figure 39). Little-to-no significant increases in cJun were noted in B virus infected macaque cells. Transcription factor cJun regulates cell cycle progression through G1 via cyclin D and cooperates with NF-κB to prevent apoptosis. It is a component of AP-1 transcription factor. Interestingly, we know from previous data (Zao and Hilliard, unpublished) that in spite of the translocation of virus-induced transcription factors regulating proinflammatory responses, there is an absence of transcription of proinflammatory genes in B virus infected human foreskin fibroblasts at five and 24 hpi, the reasons for which are unclear at present.

We next investigated cFos activation since cJun and cFos can form heterodimers to regulate specific genes. Both are associated with regulation of cell cycle and apoptosis via AP-1 regulated genes. cFos does not bind to promoter regions of these genes alone, but through binding with cJun. Again FACS analyses were performed to quantify changes in cFos levels and to compare B virus infected human and macaque cells at one and five hpi. Representative data from multiple analyses are presented in Figure 40 A & B.

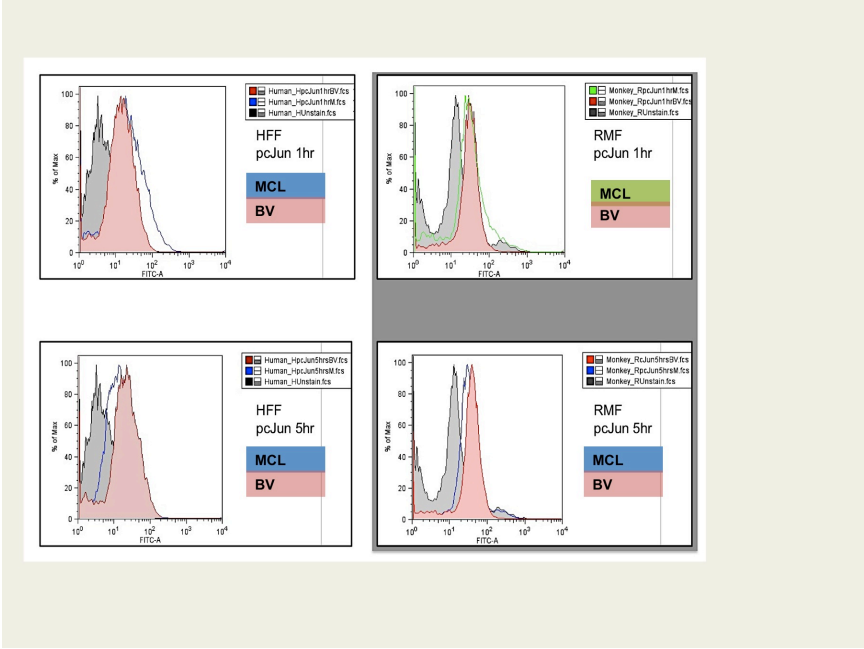


Figure 38. JNK-associated transcription factor pcJun during B virus infection at one and seven hours post infection

Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were set for 10000 events and data are representative of multiple experiments performed.

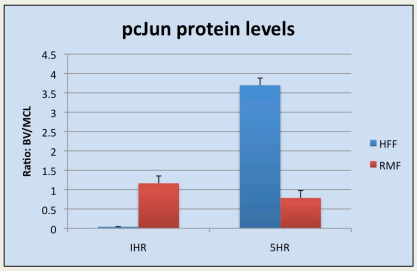
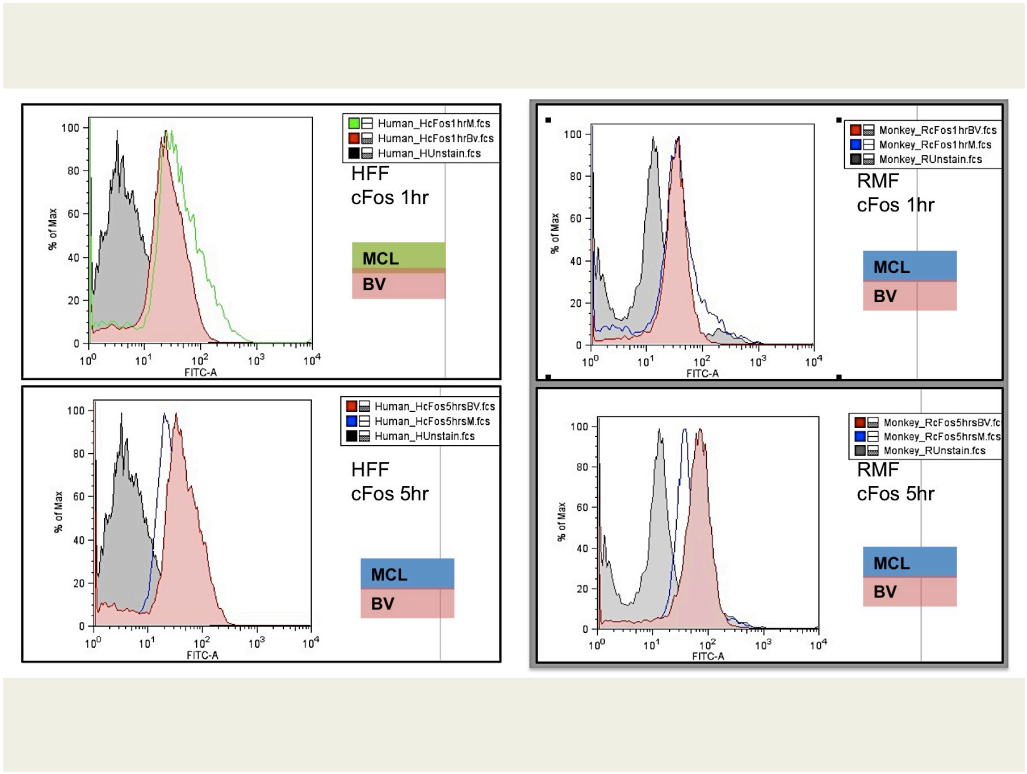


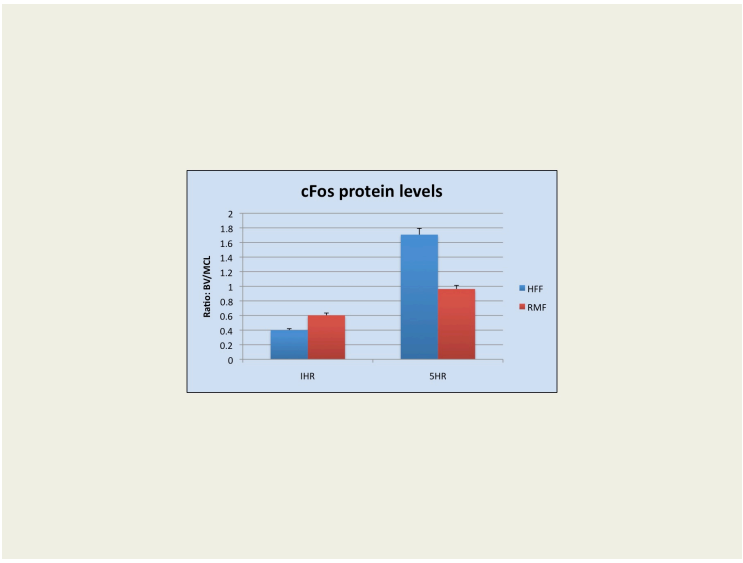
Figure 39. JNK-associated transcription factor pcJun through 5 hours post infection
Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were for 10000 events and data is representative of duplicate experiments. Data represents fold-variation between infected and uninfected cells from each species.

Interestingly, cFos levels appear to be relatively low at one hour pi in both cell lines, but by five hours post infection, levels of cFos are elevated in a virus-specific manner in human cells (Figure 40A & B), similar to what is seen for cJun. Histograms of FosB shows a similar virus-specific pattern to that of cJun with both human and macaque cells expressing lower amounts at one hours post infection and by five hours post infection there is a clear increase in virus-specific expression only in human cells (Figures 40 & 41). Comparative analyses of macaques

versus human cells can be seen in Figures 40 &41 which shows the P/N values calculated for each species using raw data from FACS analyses.



A.



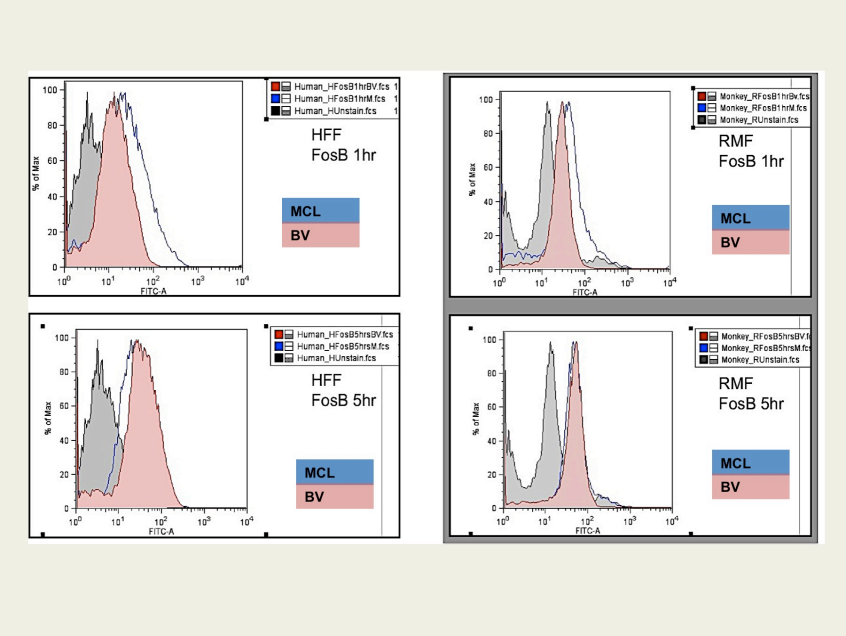
B.

Figure 40. JNK-associated transcription factor cFos after B virus infection.

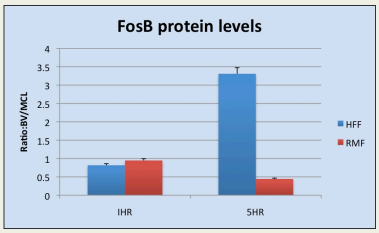
Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were for 10000 events and data is representative of duplicate experiments. A) Histograms of FosB shows a similar virus-specific pattern to that of cJun with both human and macaque cells expressing lower amounts at one hours post infection and by five hours post infection there is a clear increase in virus-specific expression only in human cells (Figure 40). Comparative analyses of macaques versus human cells can be seen in B) which shows the P/N values calculated for each species using raw data from FACS analyses.

Analysis of FosB was performed in a similar approach to cJun and cFos. FosB plays a role in transcriptional regulation as it seems that in its absence there is reduced AP-1 associated transcriptional activity. Figure 41A shows levels of FosB in B virus infected human and macaque cells, while Figure 41B shows the analysis of virus-influenced levels of FosB in each cell type. Again little increase of FosB is seen in B virus infected macaque cells while greater than a three-fold increase is seen in B virus infected human cells. The lack of transcriptional activity in B virus infected macaque cells has to-date never been observed and is curious. These data suggest that B virus employs a mechanism to dampen activation of transcriptional activity via an upstream block in transcriptional factor activation, while B virus fails to accomplish this blockade in human cells. Data from Zao and Hilliard (submitted for publication) suggests that in B virus infected human cells, transcriptional activity is suppressed by in the nucleus since transcription factors are activated and translocate to the nucleus, but fail to transcribe proinflammatory

genes. Data gathered for this dissertation show that there is a clear difference in species-specific regulation of B virus.



A.



B.

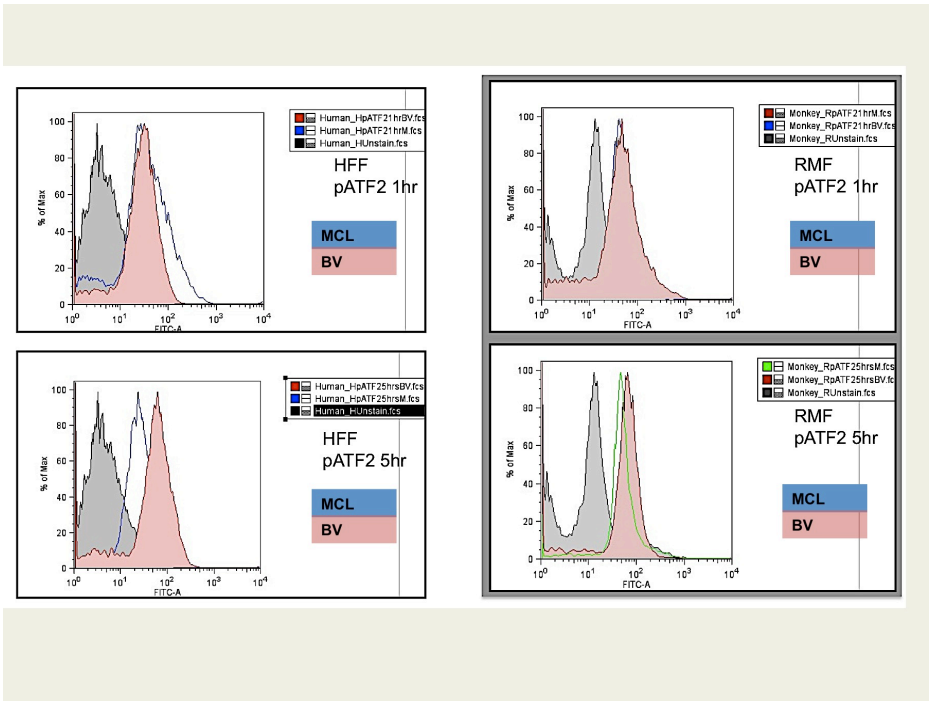
Figure 41. JNK-associated transcription factor FosB after B virus infection

Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined timepoints, medium was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was followed by primary antibody and the fluorochrome-conjugated secondary anti-

body prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were for 10000 events and data are representative of duplicate experiments.

We next evaluated ATF2 levels in B virus infected human and macaque cells using the same methodological approach. Figure 42 shows FACS data that suggests that in human cells there is an early virus-specific suppression of p38 associated activated ATF2 at one hpi, while by five hpi there is a virus-specific increase in activated ATF2, unlike the responses observed to occur in macaque cells. B virus infected macaque cells show minimal ATF2 activation at one hpi, while at five hpi there was an increase, however, significantly less than that seen in human cells. The implications of these observations will be explored by analysis of genes upregulated by ATF2. Figures 42 & 43 shows the relative fold-changes in activated ATF2 in B virus infected human and macaque cells.

A.



B.

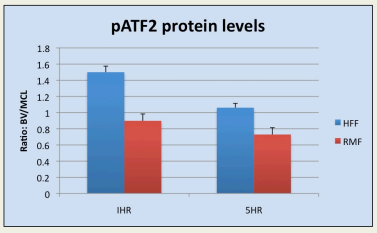


Figure 42. The p38-associated transcription factor pATF2 after B virus infection

Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were for 10000 events

and data is representative of duplicate experiments. A. histogram analysis of temporal changes in activation of ATF2 in B virus infected human and macaque cells. B. Comparative analysis of fold-changes of activated ATF2 in B virus infected macaque and humans cells.

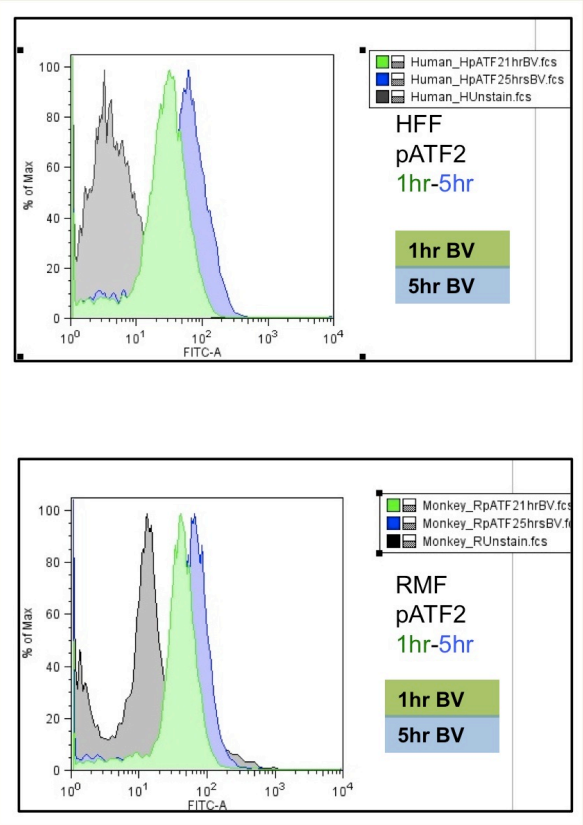


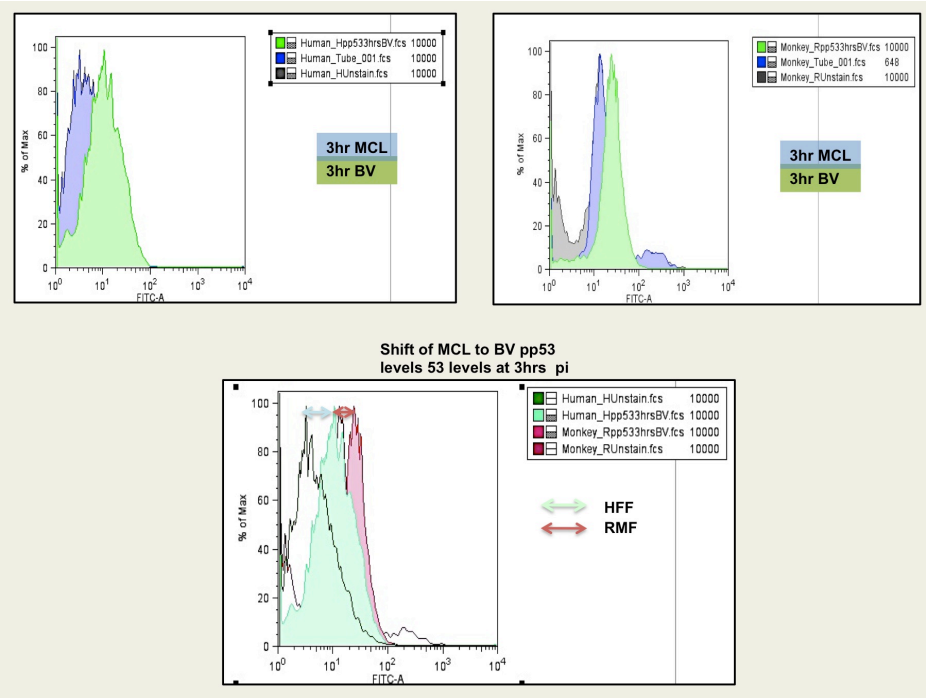
Figure 43. The p38-associated transcription factor pATF2 through 5hrs pi comparative analysis of levels at one and five hpi together for both B virus infected macaque and humans cells

Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resuspended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were for 10000 events and data is representative of duplicate experiment

ATF2 is important for the regulation of growth control functions particularly in concert with cJun by forming homo-or heterodimers. It is also a response transcription factor that is activated particularly as a result of environmental changes in the cell's microenvironment. It appears from the data shown in Figures 42 & 43 reveal that activated ATF2 is found at slighty higher levels in B virus infected humans cells relative to B virus infected macaque cells, however, analysis of data by Student T-test reveals that there are no significant differences between ATF2 activation levels between B virus infected macaque and humans cells. This may simplay suggest that ATF2 does not play an important role in the cell response to B virus.

As part of the group of transcription factors analyzed, the next experiments were designed to measure activated p53, a key element in induction of apoptotic defense responses. Although simplexviruses in general efficiently block apoptosis, there are essential components of this pathway that play a role(s) of virus replication processes. We again observed that in human cells that the relative levels of apoptotic transcription factor pp53 were greater when compared to macaque cells after B virus infection (Figure 44A). The fold-changes for each can be seen in Figure 44B which shows a three-fold increase in activated p53, perhaps as a rush for the cell to self-destruct in response to B virus infection, however activated p53 drops radically by five hpi. B virus infected macaque cells show activated p53 by five hpi. Later timepoints will be examined in a separate study to determine if the activated p53 results in effective apoptosis in macaque cells and thereby dampening virus load.

A.



B.

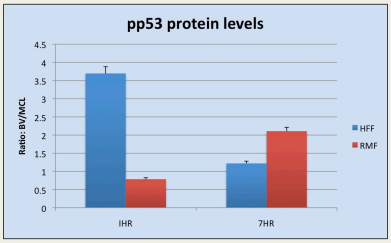


Figure 44. JNK- and p38-associated transcription factor pp53 after B virus infection
Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5, and at defined time points, media was removed and enzymatic digestion was used to remove adhered cells. This process was followed by a cold PBS wash and fixation in paraformaldehyde (PFA) 4 to 8%. Fixative was removed and cells permeabilized and resus-

pended in 100% methanol. Cells were equally aliquoted and incubated in blocking buffer; the blocking was, followed by primary antibody and the fluorochrome-conjugated secondary antibody prior to detection. The denotation M: represents mock-infected, BV: represents B virus infected and Unstain: represents uninfected unstained cells. FACS limits were for 10000 events and data is representative of duplicate experiments. A. Histograms resulting from FACS analysis of B virus infected human and macaque cells. B. Fold-changes in activated p53 in B virus infected human and macaque cells.

Comparative analysis of the selected transcription factors are shown in Figure 46 following (seven hpi) the observed increases seen in FACS analysis of B virus infected and uninfected macaque and human cells were examined. Figure 45 shows the overall relative gene expression mRNA levels of transcription factors at 7 hours post infection. At this time point, we observed in B virus infected cells that there was a >3-4-fold increase in the levels of the investigated transcription factors cJun, cFos, and FosB, and two-fold increases in cc3 and p38 whereas no significant increases were found in B virus infected macaque cells other than a two-fold increase in activated p53. Upregulation of these transcription factors are partially self-regulated and thus, increases reflect the drop in activated transcription factor to replenish the supply so that the cells are prepared for the next need to respond.

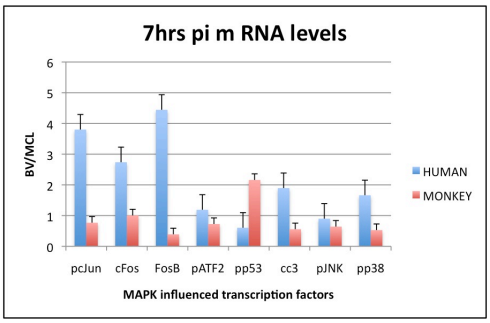


Figure 45. The gene expression levels of transcription factors influenced by JNK and p38
Human (HFF) and monkey (RMF) cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5 and at defined time points, media was removed and RLT buffer (Qiagen) added for total RNA. RNA was then purified using Qiagen RNeasy and RNeasy Plus Kit, followed by cDNA conversion using SA Biosciences RT^2 First Strand Kit. Analysis was then done using by PCR. Results are BV/MCL for the stated protein and bars represent the mean ± standard error of the samples.

6.3.2 Cytokines involved in shaping the innate and adaptive response

Each of the transcription factors evaluated play a key role in upregulation of specific cytokine responses that ultimately shape the type of adaptive responses of a host in response to infection. Cytokine secretion is a key event of the innate immune response to infection and plays an important role not only in shaping the adaptive response to infection, but also the recruitment of innate immune cells to the site of infection. The types and amounts of cytokines secreted after infection differ among cell lines and cell types, for example cells of the lymphoid

origin, once recruited, secrete abundant amounts of specific cytokines in response to infection. However, this cell type dependant secretion includes cells within the region of the primary infection that play an important role in the initial secretion of cytokines to influence surrounding tissue and circulating immune cells.

The following figures (Figure 46 through Figure 47) show the relative kinetics of proinflammatory cytokine secretion after B virus infection of human and macaque cells. Do cells of either species successfully induce specific cytokines to control virus replication or the effects of this replication. And in order to evaluate the effects of the MAPK pathway activation, experiments were designed to investigate whether B virus infection in the presence of MAPK inhibitors derailed the cytokine responses observed. Macaque and human cells were infected as previously described and at different timepoints after infection, supernatant fluids were harvested to measure. Luminex multiplex technology was used to perform supernatant evaluations for specific cytokines measurements. Figure 46 shows the results of IL2 that we observed in the B virus infected human and macaque cells. By comparison, the same values are shown for cells exposed to mock-infected (uninfected) cell lysates. These results show that little-to-no virus-specific IL2 is being produced at any timepoints (1,5,7 hp) by either cell species following infection with B virus. This was not an unexpected observation since previous experiments performed using microarray analyses of B virus infected human cells showed essentially the same results. The experiments performed during the course of this dissertation show for the first time that B virus infected macaque cells also fail to produce IL2.

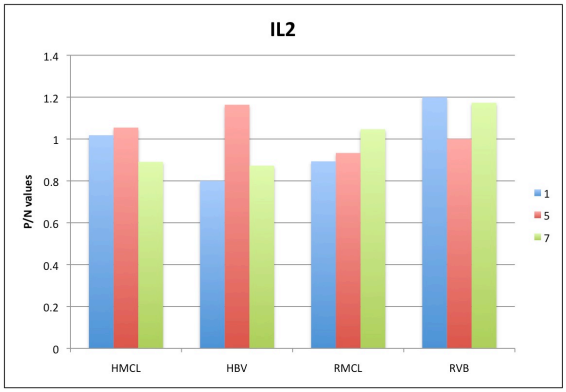


Figure 46. Interleukin 2 (IL2) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. P/N values were calculated as a ratio of the optical density values of B virus infected cell supernatants/ cytokine specific negative control provided by the manufacturer.

Representative IL-4 measurements shown in Figure 47 suggested that in B virus infected monkey cells there was a four-fold change greater than that of the mock at 5 hours post infection. Whereas in the human cells the relative levels of IL4 were unchanged at all times post infection. This is an interesting finding that suggests that B virus infected macaque cells can recruit immune responder cells that may play a role in controlling infection at some level. Future experiments will be performed to determine the effects of IL-4 on effector cell population recruitment and the effects of these cells on virus load. Whether the monophasic increase in IL4

is enough to influence recruitment of additional innate response cells and/or adaptive responders requires further study.

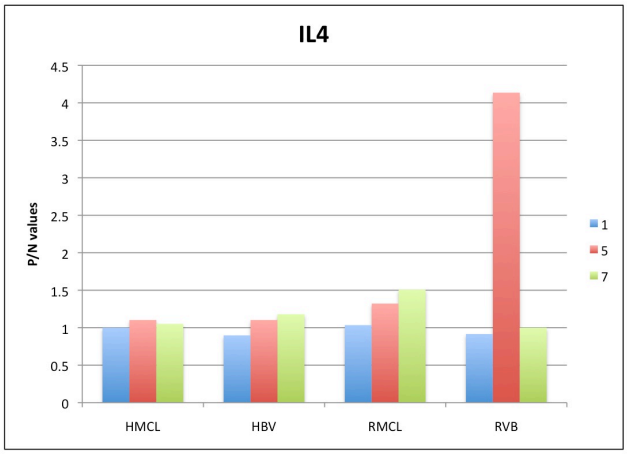


Figure 47. Interleukin 4 (IL4) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

In Figure 48 we observed that after B virus infection of human cells IL6 was produced , albeit at low levels. Macaque cells showed no virus specific increase in IL6, but interestingly levels of IL6 were elevated relative to the manufacturer's negative control, suggesting that IL6 levels were present in relatively high levels in macaque cells exposed to virus or simply cells

lysates free of virus. Anecdotally, macaques appear to have high levels of serum IL6. The significance of this is unknown. These levels may be effective for recruitment of neutrophils, which will be explored in later experiments.

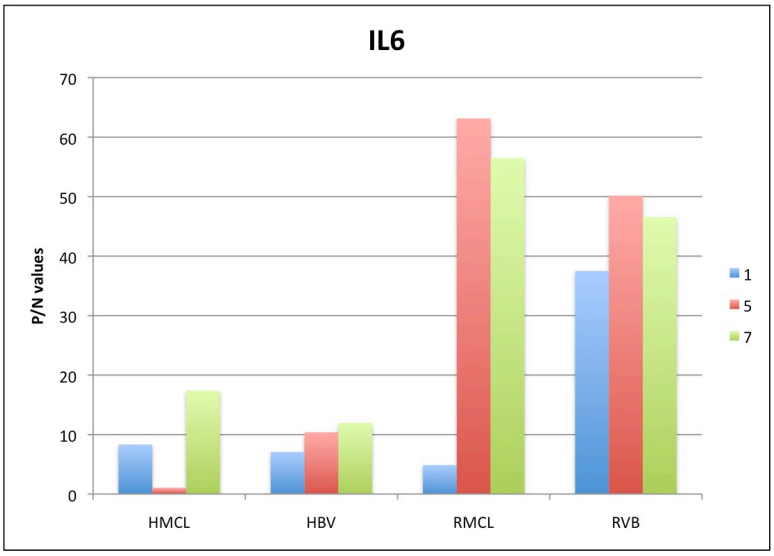


Figure 48. Interleukin 6 (IL6) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

In figure 49 we observed that there were no significant differences by Student t-test analysis between B virus infected human and macaque cells with respect to production of IL8.

Again, this data supports data from microarray experiments using B virus infected human cells and presents the first look at B virus infected macaque cells. Thus, far, B virus infected human and macaque cells are not responding to recruit proactive defenses, even though we have observed that specific components of the MAPK pathways are engaged by the infection, albeit differently in each cell species.

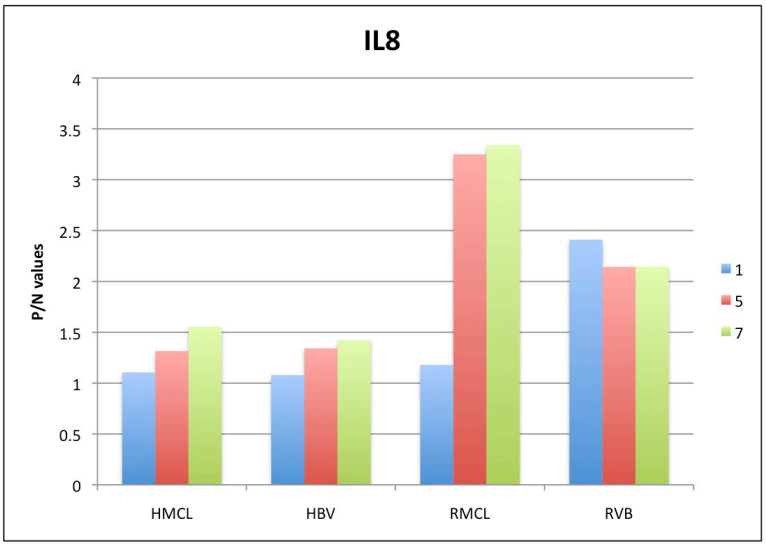


Figure 49. Interleukin 8 (IL8) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

In figure 50 we observed that the levels of IL10 were relatively unchanged in the human and macaque cells after B virus infection when compared to their mock. Less than 2-fold changes were observed in levels of IL10, which is unlikely to result in physiological response. Failure of the immune response recruitment at this stage of infection is thus not due to production of IL-10. In future experiments, other potential immunosuppressor molecules will be examined.

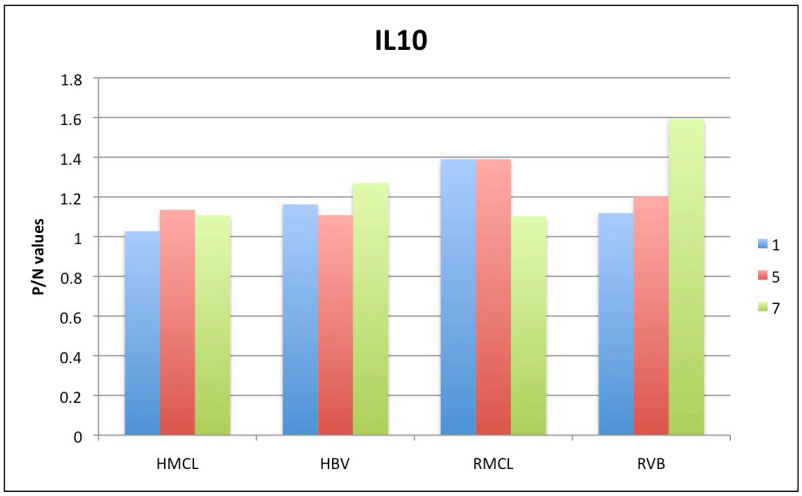


Figure 50. Interleukin 10 (IL10) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for

LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

In figure 51 we observed that there was a 2.5-fold increase in the IL12 (p70) levels in mock-infected human cells, but not in virus infected human cells. This observation suggests that there is a potential virus down-modulation of IL12 (p70) that was less than that of the mock at 5 and 7 hours post infection. However, in the monkey cells there was a relative increase in the IL12 (p70) levels at 5 and 7 hours post infection**.** It is unlikely that these modest fold-changes will result in significant levels of IL12 (p70), but again, determination requires further study.

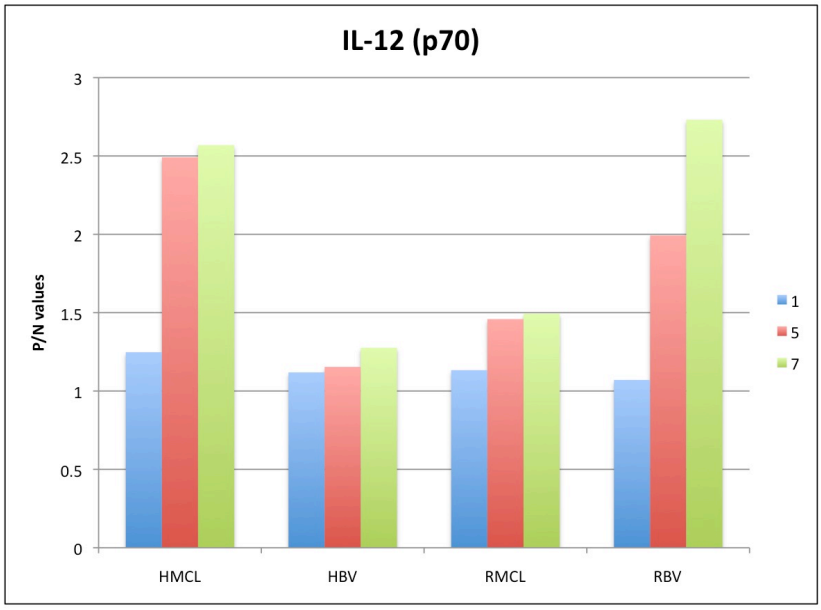


Figure 51. Interleukin 12p70 (IL12p70) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

Interferon-gamma is perhaps one of the most important antiviral proteins. In Figure 52 we observed no significant differences between B virus infected macaque and human cells, nor between these cells and cells treated with uninfected cell lysate, indicating that B virus infection does not upregulate IFNα in either cell species. Again this observation corroborates previ-

ous observations made with B virus infected human cells and suggests that in this regard B virus infected macaque cells show a similar absence of IFNα production.

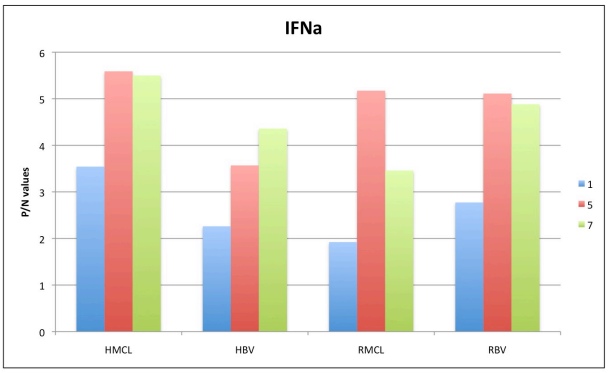


Figure 52. Interferon alpha (IFNα) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour, unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for-LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings.(See Materials and Methods)

Analysis of B virus infected macaque and human cells for the production of IFNγ indicates that virus infection of either cell species fails to induce IFNγ (Figure 53).

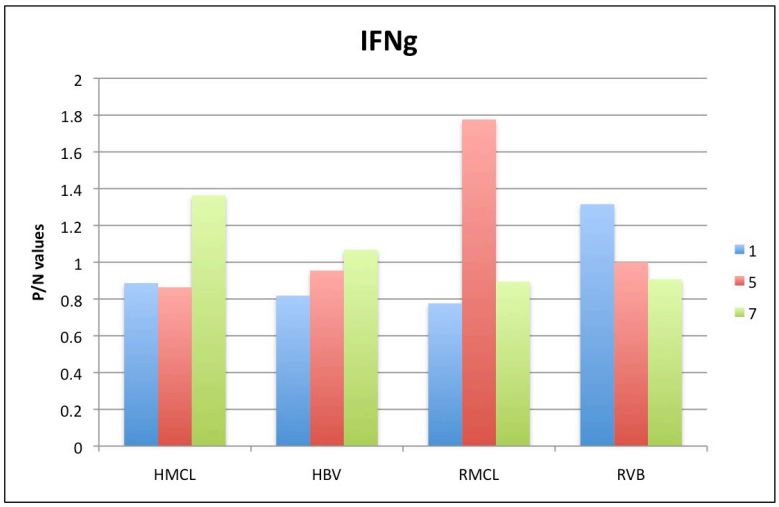


Figure 53. Interferon gamma (IFNγ) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

MCP1 plays an important role in the recruitment of monocyte defenses and previous studies in our lab have shown that B virus infected human cells fail to recruit monocytes in a transwell system. Data analysis shown in Figure 54 also shows that B virus infection of macaque cells induces no more MCP1 that treatment of cells with uninfected cell lysates as seen by barely a two-fold increase in MCP1 levels after the first hour post infection. B virus infected

humans cells may actually downregulate MCP1, but further experiments must be done to verify that this is significant.

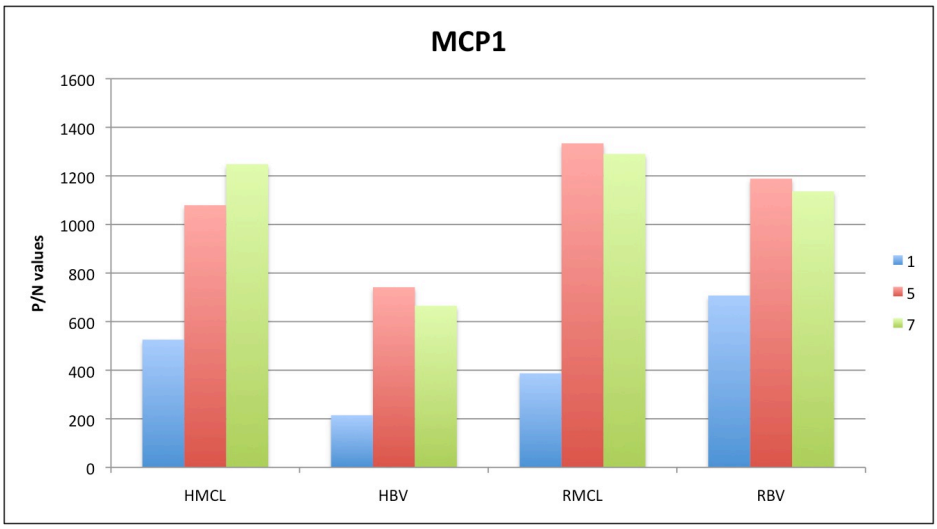


Figure 54. Monocytic chemotactic protein 1 (MCP1) levels after B virus infection
Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. (See Materials and Methods)

Data shown in figure 55 reveal no significant changes in GM-CSF levels. Cummulatively, each data set is substantiating that there is a pattern in both B virus infected macaque and human cells of no significant cell responses. Cells from either host appear to fail to recognize virus invasion is progressing. The stealth nature of the infection is curious. What role(s) is induction of the MAPK pathway playing? To address this question, the following experiments were conceived and performed.

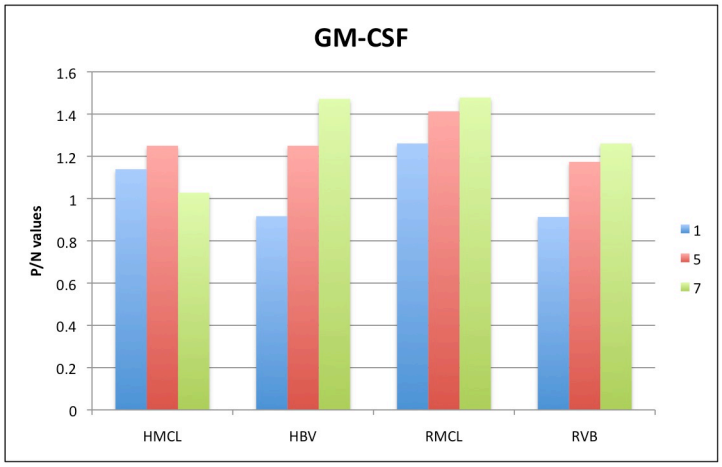


Figure 55. Gramulocyte-macrophage colony-stimulating factor (GMCSF) levels after B virus infection

Cells were either mock-infected (MCL) or infected with B virus (BV) at MOI 5. After 1 hour unadsorbed virus was removed and media replaced with minimal volume. At defined time points, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for

LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. Results are the mean ± standard error of the samples. (See Materials and Methods)

6.3.3 Cytokine profile after infection in the presence of MAPK inhibitors

Having observed that the MAPK p38 and JNK are activated, but fail to trigger significant upregulation of defensive responses, i.e., cytokine production in B virus infection, I investigated how the inflammatory cytokine profile changed in the absence or the presence of the MAPK by the use of inhibitors and stimulators of the MAPK pathway.

The results (Figures 56 and 57) show that inhibition of the MAPK-JNK led to a relative increase in secreted IL6 in both cell lines. In contrast, p38 inhibition led to a relative, but poor increase in the IL6 levels in B virus infected human cells and barely a two-fold increase in B virus infected macaque cells. Stimulation of the p38 and JNK pathways with sorbitol lead to relative increases (2.5-fold) in IL4 in B virus infected macaque cells.

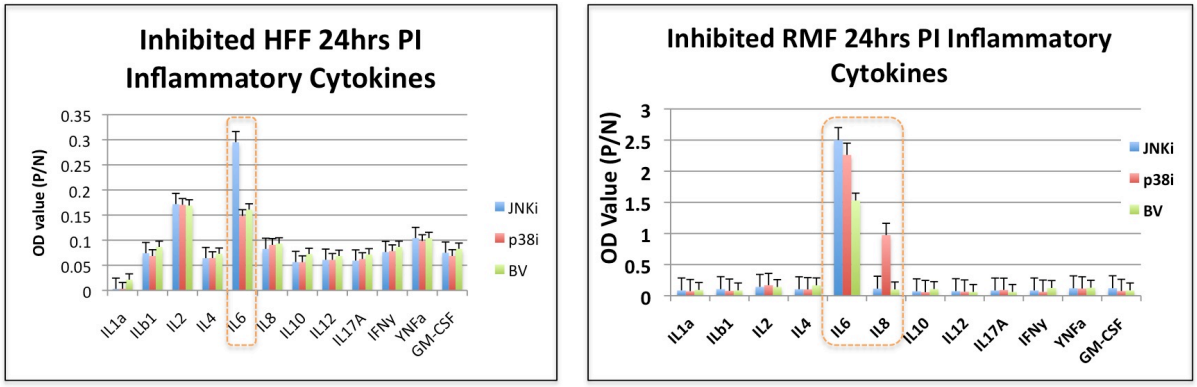


Figure 56. Cytokine profile after B virus infection in the presence of JNK and p38 inhibitors
Cells were infected with B virus (BV) at MOI 5 in the presence or absence of the stated inhibitor. After 1 hour, unadsorbed virus was removed and media replaced with minimal volume with stated inhibitor (JNKi L form for JNK inhibition and SB202190 for p38 inhibition) added to media. At 24 hours post infection, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. Results were calculated as: (BV + inhibitor)/BV only, for the stated protein. Bars represent the mean ± standard error of the samples.

6.3.4 Cytokine profile after infection in the presence of stimulators

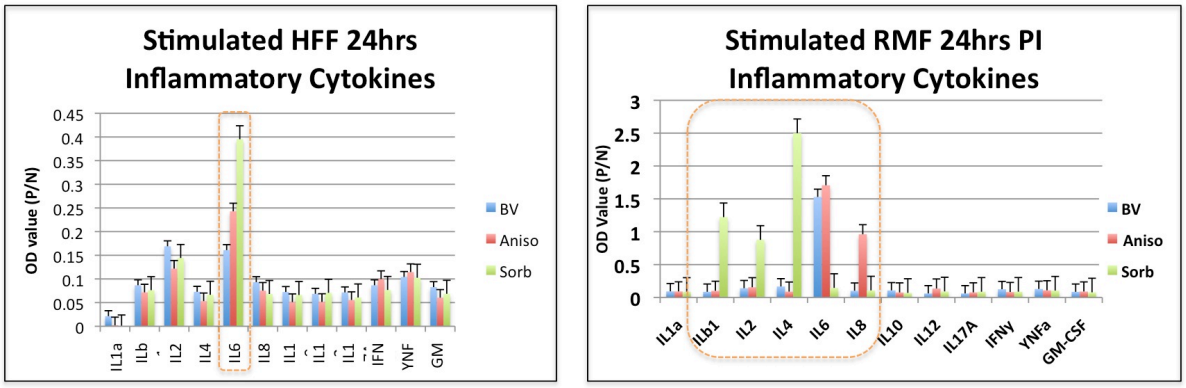


Figure 57. Cytokine profile after B virus infection in the presence of JNK and p38 stimulators
Cells were infected with B virus (BV) at MOI 5 in the presence or absence of the stated stimulator (Anisomycin for p38 stimulation and sorbitol for JNK stimulation). Note that though each stimulant is directed to a specified MAPK (p38 or JNK) they have both been known to affect the levels to p38 and JNK in a stimulus and cell types dependant manner. After 1 hour, unadsorbed virus was removed and media replaced with minimal volume with stated stimulator added to media. At 24 hours post infection, supernatant was removed and 10% Tween and 10% DOC added. Supernatant was then vortexed and cytokines read using the Luminex 100™ IS, 200™/HTS by Luminex Corporation for LiquiChip analysis or using the ELISA plate reader as specified by manufacturer for desired OD readings. Results were calculated as: (BV + stimulator)/BV only, for the stated protein. Bars represent the mean ± standard error of the samples.

7 Discussion and summary

7.1 Host specific model of infection

The first anatomical site for virus replication - prior to the retrograde transport of virus by sensory neurons innervating the region - is the skin. The skin functions as an external barrier, and one of its many layers is the dermis where fibroblasts comprise the majority of cells. Fibroblasts play an important role in skin innate immunity and are capable of secreting many cytokines, chemokines and antimicrobial peptides. The outcome of these responses direct the recruitment of the cellular responders, e.g., neutrophils, macrophage, dendritic cells, natural killer cells and possibly others. The collective innate responses direct the specific adaptive responses seen associated with disease pathogenesis in specific hosts.

By establishing a cell culture with cells representative of the majority cell type after infection in each host, we can investigate and dissect out the initial molecular sensing of B virus and downstream factors associated with and involved in the activation of the MAPK pathway which in turn upregulates specific genes putatively leading to cytokine secretion and the induction of apoptotic events that can potentially be subverted for successful replication, assembly, and egress of virus. A major benefit of the natural and foreign host cell model system is the ability to infect both cell types with B virus and observe whether there are host specific differ-

ences or similarities that may correlate with the observed different outcomes in pathogenesis (death in foreign host and survival in natural host). Thus, the model potentially allows for examining early, local, host responses that influence the outcome of the infection after B virus-host interaction occurs. The virus (B virus) was my tool and allowed for a comparative investigation of what host-dependent changes were occurring at early times post infection. The ability of both cell types to produce infectious virus though 24 hours as analyzed by plaque assay, and to express US11 and viral glycoproteins gC and gD, suggests that B virus is able to replicate during the initial round of replication in these primary cells from different hosts. The levels of infectious virus and viral proteins with increased rounds of replication though not investigated in this dissertation (as all times investigated were within one round of replication) may show differences in a cell-type dependent manner and may become more magnified with increased rounds of replication. This effect could also have an influence on the overall host-dependent responses observed as the levels of infectious virus present at the initial site of infection can be directly related to the ability of the virus to evade host responses and enter the PNS or CNS, or perhaps lower replication efficiency contributes to a temporal delay in neuroinvasion. With the ability of B virus to replicate in both cell types albeit with different efficiencies of infection our data suggest that the both B virus infected macaque and human cells fail to deploy innate defense responses unlike cells infected with HSV-1. Perhaps most interesting, is that macaque and human cells accomplish this by two distinct mechanisms. Macaque cells fail to activate transcription factors, while humans cells fail to have activated transcription factors successfully upregulate proinflammaory genes they regulate.

7.2 Identification and analysis of differential signaling

This research was focused to investigate underlying reasons for different host outcomes in response to infection and determining what role, if any, is played by one pathway (MAPK) that is known to play an important role in the innate immune response to infection. To accomplish the studies proposed at the outset of this research, a novel cell line from macaques had to be established. These cells needed to be fibroblasts in order to perform studies in parallel in cells from macaque and human sources. The establishment of macaque fibroblast cells was labor intensive, but ultimately successful and this success enabled a comparative analysis B virus infection in the primary target cells of B virus when it infects either host. It is these target cells that provide sufficient virus for populating dorsal root ganglia via retrograde travel through sensory axons of the peripheral nervous system and it these cells that determine whether effective innate and adaptive immune defenses are successfully engaged following B virus infection. There is no animal model currently available to study B virus pathogenesis. One of the goals of the studies described in this dissertation was to gain insight into molecular correlates of infection that may lead to the establishment of susceptible transgenic mice that can serve as *in vivo* model of B virus infection.. But in the meantime, the question presenting at the onset of this research is can we successfully establish a cell culture model of infection to gain understanding about the dynamics of B virus infection (Figure 28) in macaque and human cells? Our results showed that each cell line supported B virus replication with kinetics previously described and published. With this data, we proceeded to analyze the initial events occurring in each cell type following B virus infection.

The established cell model system was subsequently used to evaluate the initial cellular responses following attachment and adsorption of virus on target cells that would be the first to encounter virus *in vivo*.. Using the cells of macaque and human origin, we analyzed post adsorption signaling cascades, specifically the MAPK pathway. The pathway begins by assembly of a complex of activated adaptor proteins that build a scaffold from with the signaling cascade begins. They (adaptor proteins) also serve as points of convergence within a cell for signals to be integrated prior to the divergent relay of the received signals to cellular signaling pathways, such as the MAPK pathway. Their complex formation and targeted pathway differs with varied cell types and pathogens encountered. Adaptors MyD88, TRIF and TRAF 6 represent three adaptor proteins known to be play a role in upstream MAPK pathway activation. Our results showed that the TRAF2 was not activated in a virus-specific manner, although TRAF6 was activated in human cells within 30 minutes post infection. No activation of TRAF6 was seen to occur in a virus-specific manner in macaque cells, although exposure of macaque cells to either virus infected cells lysates or uninfected cell lysates did result in activation of TRAF6 and TRAF2. It would appear that membrane perturbations are sufficient to initiate the signaling via TRAF2 and TRAF6.

As previously mentioned, the MAPK pathway represents one of several pathways known to be activated in response to infection. We observed that virus-independent activation of JNK1 and JNK2 occurred in both species of cells. JNK3 was also found to be activated which may be a novel finding for fibroblasts as this isoform of JNK is usually found in heart, brain and testis rather than in cells derived from skin. JNK is known to be associated with p53 in cells, leading to p53 targeted degradation, the regulation of JNK1 and JNK2 have been shown to have opposing

roles in a cell type dependent manner in fibroblasts and the stimulus and duration of JNK signaling determines whether it is a pro-survival signal or an apoptotic inducing signal (Fuchs, Dolan et al. 1996; Fuchs, Adler et al. 1998). JNK isoforms have also been shown to regulate the half-life and stability of cJun, whereby JNK1 tends to activated cJun and JNK2 tends to have a greater affinity for cJun and thus target it for ubiquitination and degradation (Fuchs, Dolan et al. 1996).

Regarding p38, in the human cells there was a steady state of activation post infection, that was virus-dependent, whereas in the monkey cells a virus specific activation was noted to occur only at three hpi. . Since both the JNK and p38 pathway can be activated by similar upstream adaptor complexes with areas of convergence and divergence to give specificity to the signaling, it is plausible to think that the kinetically different times of JNK and p38 activation may be related to the kinetics of viral gene expression in each cell line, such that one class of viral genes may trigger one pathway at an earlier time. It is also plausible that transcription factors such as AP1, known to be regulated by the MAPK are being upregulated in the human cells to allow for a productive infection as some viruses, inclusive of the *herpesviruses* are known to have AP1 binding sites in the promoter regions for some of their early genes (Xie, Pan et al. 2005).

Although we could not determine if the MAPK response was following a MyD88 or TRIF mediated path to activation, the use of inhibitors to either p38 or to JNK showed that both p38 and JNK were influential in the relative amount of viral replication that occurred in each cell line. MAPK p38 and JNK were essential for B virus replication in the human cells, whereas pp38

appeared to be dampening virus replication in the monkey cells. As shown in the results, these inhibitors had varied mechanisms of action and it should be noted that those that competed with the ATP binding sites had relatively larger effects on virus replication, suggesting that they catalytic ATP binding site plays a primary role in p38 and JNK activation. Regarding both JNK and p38 inhibition during virus replication, it may be that one of the pathways, p38 or JNK, can compensate for the other, or that it is the balance between these two factors that ultimately determines the overall fate of the cell after infection in a host-dependent manner (Hsu, Saffran et al. 2005).

The induction and stabilization of regulatory proteins, such as the inflammatory cytokines, transcription factors and growth factors, that can act as cis signals to promote mRNA degradation and regulate translation are important events in host cells. These functionally diverse proteins tend to have an AU-rich instability element (ARE) in their 3' untranslated region (UTR) and ARE stabilization has been shown to be associated with MAPK (p38) induction, with the regulation of cell death and with ND10 domains (Chen, Del Gatto-Konczak et al. 1998; Corcoran, Hsu et al. 2006). Thus, the observed increased in p38 activation after infection may also play a role in gene regulation and stabilization by ARE mRNAs in a cell type dependent manner to promote viral replication.

7.3 MAPK-dependent apoptosis

The SAPKs JNK and p38 are multifunctional kinases that influence numerous cellular events in response to stress (viral infection) such as cell survival apoptosis and cytokine secretion. The MAP3K-ASK1 has been implicated in the stimulation of apoptosis via the JNK and p38

pathways. This research not only showed that we have cell type specific responses that are not only kinetically different but also some responses that are virus specific, such as ASK1 induction. The increased ASK1 in human cells at earlier times that monkey cells suggests that ASK1 a MAP3K may be playing an early role in the induction of MAPK-dependent apoptotic events. Because PKR and ASK1 are known to interact and their interaction can lead to p38 and JNK depended cell death, it is plausible that though we see virus specific ASK1 activation in both hosts, the knowledge that PKR activation if inhibited by Y34.5 in B virus infected cells implies that this association for ASK1 mediatesd apoptosis is inhibited.

The roles of the MAPK p38 and JNK have been varied one such role may be involved with modulation of apoptosis. Typically, the activation of JNK is observed in conditions involving cell survival and the inhibition of apoptosis. However, JNK has also been shown to be activated in situations where the induction of apoptosis occurs and the interaction of active JNK with p53 leads to pp53 being released from JNK allowing for downstream protein responses such as caspase 3 activation to occur (Sumbayev and Yasinska 2006). Regarding p38, most studies refer to it as a pro-apoptotic inducer of events prior to caspase activation, and p38 has been shown to destabilize the survival protein BCL2 mRNA levels. However, it should also be noted that p38 activation has been linked to increased viral yields in the absence of p38-mediated BCL2 destabilization suggesting that the roles of p38 and JNK in cells are dependent on stimulus-duration type, cell type and stimulus type (Zachos, Clements et al. 1999). Our results also support this notion as we observed a virus specific increased numbers of human cells expressing p53 whereas a non-virus specific relative increase was observed in the monkey cells. The executioner caspase, cleaved caspase 3 also showed a relative bi-phasic increase after infection.

However, as we know that a productive infection is established in both cell lines, this ability to have successful replication must indicate that cell death/apoptosis is inhibited or limited at some stage in the replicative cycle.

Overall the MAPK -influenced events investigated in this study do not suggest that induction of apoptosis is not occurring, so where the block occurs is yet to be determined and future investigates will look at DNA cleavage (Tunnel assay) and PARP levels, both events which indicate terminal apoptosis. Recent work has shown that some viruses are able to use caspases to cleave their viral proteins, to act as facilitators of protein nuclear transport and in the regulation of transcription (Bloom 2004). However this is a host-cell type and pathogen-dependent observation. The activation of caspase 3 cleavage even with virus replication may suggest a role for it in the replication of B virus (though that has not been determined) in these cell lines.

Stimulation of the pathway in human cells also led to a JNK3 isomeric protein level increase. This finding is important as knockout studies of each JNK isomer have shown that JNK3 plays an important role in neuronal cell death (Loss of JNK led to protection of brain-derived neurons) (Bogoyevitch MA 2004). The role of the isomer in B virus infection was not determined, but published studies have suggested that the JNK isomers can play different roles in viral infection, and that the cross-talk between the MAPK pathway and the NFkB pathway plays an important role in determining cell fate (Wen-Ming Chu 1999; Salvatore Papa 2004). All three JNK isoforms have also been shown to regulate p53 by phosphorylation and this post-translation modification by the JNK1 isoforms can lead to different outcomes (Tafolla, Wang et al. 2005). Previous work in our laboratory has shown that the NFkB pathway is activated early

after infection in human cells (foreign host), though transcription factors associated with the pathway are unable to bind to promoter regions and influence gene transcription (Zao 2007). Thus, the overall suppression of the NFKB pathway may be a contributing factor to the observed increase in MAPK activation (especially in the human cell line). JNK regulation of both pro-apoptotic and anti-apoptotic proteins most likely has to do with a balance of the two opposing signals after JNK activation.

7.4 Early innate cytokine correlates in response to B virus infection in both hosts

The modulation of gene transcription in the nucleus in response to changes occurring in the cell due to viral infection allows for proteins involved in the innate immune response to be upregulated. This upregulation of transcription factors regulated by MAPKs involves the Jun, Fos and ATF family of proteins. The data showed that the for the human cells there was a general trend for a greater number of cells to express the FosB and ATF2 transcription factors at relatively greater levels when compared to the monkey cells. This observed expression may be linked to transcription factors use by the virus to enhance its own replication as the promoter regions of the immediate early or early genes of some viruses, inclusive of B virus, have AP1 binding sites (as mentioned previously). Thus, the overall initial upregulation of AP1 factors in both hosts can play an initial role in the regulation of genes involved in the innate immune response to the infection and also in virus replication as it manipulates cellular processes and exploits upregulated transcription factors.

Of the many cytokines investigated at one through seven hours post infection, there appeared to be a greater potential for stimulation and development of a Th2 mediated response

versus a Th1 mediated response in macaque cells, whereas the response in human cells did not appear to lean to either a Th1 or a Th2 mediated response as very few cytokines were detected. Members of the Th1 interleukin (cytokines) family are IL2, Lymphotoxic protein (LP), IFNγ, TNFβ and they are generally thought to activate macrophages to cell mediated immunity. This is different to the Th2 interleukin family whose prominent members are IL4, IL5, IL10 and IL13. These Th2 cytokines play a role in host antibody-mediated responses and can inhibit macrophage function (Spruance, Evans et al. 1995). Differentiation of cells involved in the early response to infection is guided by the cytokines found or secreted in the surrounding local environment. As such the early increase in IL2, IL6, IL7, IL8, IFNα, IFNγ, and MCP in monkey cells suggests that though a proinflammatory response is initiated early after infection, key players that directly shape the response to a Th1 or a Th2 response are also upregulated allowing for CTL responses that include: PBMC induction to secret IFNγ; natural killer (NK) cell and T cell secretion of cytokines; increased NK cell activity; dendritic cell (DC) priming for IL12 production; macrophage activation, chemo-attraction of monocytes, DC and NK cells and enhancement of MHC expression in professional and non-professional cells. With the upregulation of IL10, a common damper of proinflammatory responses that has also been shown to have stimulatory capabilities, occurring at late times post infection, this suggests that its upregulation could be involved in not only decreasing the levels of proinflammatory cytokines but also limit antigen-presenting cell functions and the expression of chemotactic molecules for monocytes.

In the human cells, only small relative increases in the levels of IL2 and IL4 early after infection and GM-CSF later in infection, could be seen. IL2 and IL4 have opposing roles such that IL2 drives a CTL response to promote Th1 response, whereas IL4 promotes a Th2 response. GM-

CSF plays a role in stimulating antigen-presenting DC to increase their functional capacity, macrophages and granulocytes. The increased levels in GM-CSF in conjunction with opposing roles played by increased IL2 and IL4 levels suggest that the human cells are also able to mount a response but it is not one that is committed or directed to a Th1 or a Th2 mediated response. As such this delayed secretion of necessary cytokines to not only contain or inhibit the infection, but also limit viral spread and shape the adaptive response can be responsible for the lack of a response generally seen in human patients who have succumbed to the infection.

Noteworthy are the upregulation of IL7 and IL12 in macaque cells. IL7 is capable of down-regulating an early critical repressor of cytokine signaling, SOCS3, allowing for amplified cytokine production (IFNα/β), increased T cell effector function and numbers and viral clearance (Pellegrini, Calzascia et al. 2011). SOCS3 has been shown by previous work in our laboratory to be downregulated after infection with B virus in human cells (Zao 2007). Whether there is a downregulation of SOCS3 in macaque cells, and what the role (if any) is played by the observed increase of IL7 levels post B virus infection in the macaque cell line is still to be determined. The increase in IL12 levels coincides with the increased levels of pro-apototic factors seen earlier and may be a late indicator that though the human cells are trying to induce apoptosis as a survival mechanism for the host, this induction occurs beyond the 3 to 5-hour window considered important for the prevention of apoptosis in *herpesviruses* (Aubert and Blaho 1999). These events are summarized in Figure 60.

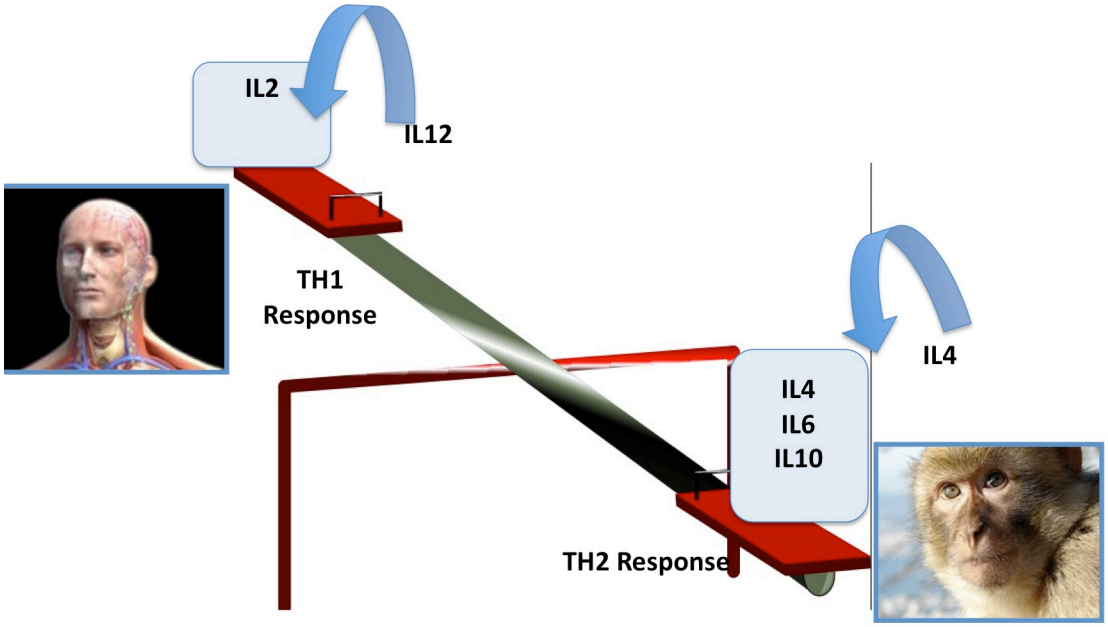


Figure 60. Host-specific summary of cytokines and chemokies after B virus infection

Collectively, these events allow for an early response that is not only mediated by early proinflammatory mediators to occur but for early shaping of a long-term adaptive response. As the development of a Th1 versus a Th2 response leads to development of specific adaptive responses and different pathological outcomes when disregulated, it is important to look at the Th1/Th2 paradigm of secreted cytokines after infection and how it play a roles in protection and pathogenesis for vaccine development and therapeutic interventions. Generally Th1 responses function to drive cell-mediated immunity whilst the Th2 responses influence the IgM to IgG and IgE antibody-mediated response.

The present study was not without its limitations and though each MAPK (JNK and p38) has a role and can influence several events including the transcriptional program within the cell after infection. The effect observed in the efficiency of viral replication and the levels and types of cytokines secreted in response to the infection, suggests collectively the contribution of type differences and other factors influence the overall outcome of the infection. It is plausible that the effects of factors influenced by the pathway are being inhibited by the IE and E viral proteins that alternate downstream targets of p38 and JNK for efficient replication are being affected; that the importance of the transcription factors upregulated in a cell-type and time-dependent manner are masked by the culture system used and that the MAPK network, which is known like other pathways to have areas of cross-talk coupled with redundancy for regulation, is robust enough to mask the true effect of inhibition of a select arm of the pathway. Also, though each inhibitor has specificty regarding their mechanism of action, care must be taken as research has shown that p38 inhibitors can activate or influence the levels of activated JNK and as such care must be taken in extrapolating information from inhibition the studies. However, with the development of peptide based inhibitors and allosteric modifiers of activated p38 and JNK (rather than ATP competitors) one may be able to not only have better targeting in inhibition but also increase efficacy.

7.5 Potential benefit of the investigated events

Virus infection leads to defined responses that are host-dependent and cell-type-dependent. The infection leads to numerous manipulations within cells such that mRNA, proteins, metabolites, channel activity and potentials as well as cell scaffold structures and membranes are changed. All of theses changes can be characterized as a molecular signature. The

changes are a biochemical signature of the infection and they will have certain alterations that are restricted to the pathogen in a stated cell type. By investigating these signatures and dissecting the role of the pathways involved, we will not only be able to develop technology that can decipher what pathogen a cell type or person has come into contact with (diagnostic application) but also be able to define types of proteins involved in specific biochemical changes that are either beneficial for the virus or lead to detrimental effects within the host.

Also this work highlights the importance of MAPK signaling post infection and the host dependent role factors regulated by the MAPK have in B virus infection. More importantly, the observed influence of inhibition on relative increases in proinflammatory cytokines IL6 and IL8, again suggests that the MAPK p38 and JNK may either directly or indirectly influence cytokine secretion and progression towards apoptosis in host cells. The observed p38 dampening of the replication in the natural host, and reduction of infectious virus in the foreign host after p38 inhibition points to a novel area not previously considered for therapeutics to B virus infection to be investigated. The propagation of signals that lead to a TH1 or a TH2 mediated response early after infection, can be a means for host survival and though we generally associate TH1 responses with antiviral clearance early after infection, it may be that the host survival is dependant on the TH1 and TH2 balance such that the monkey (as shown in the data) is able to express factors associated with both responses and as such has cellular immunity and humoral immunity to control/limit the infection and spread of B virus.

The overall induction of MAPKs and role of MAPKs in B virus infection has been shown in this study to occur in a host dependent manner, and though not all induced events are virus

specific, the study highlighted the importance of MAPK's in virus replication and cytokine secretion, as summarized in Figure 6.48. This regulation and modulation involves a constant shift in the balance of transcription factors and cytokines such that their net effect is dependant on the time of release, the location of the effect, the responsiveness of cell within the area of the infection, the density of receptors for the region of its effect and the overall composition of other competing or synergistic cytokines within the defined area. As such, future studies to address each of theses areas and how the interaction of primary fibroblast cells with other circulating cells found in the area post infection to limit replication and spread will assist in dissecting the overall mechanism by which the natural host is able to control the infection and survive.

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