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The Impact Of Interferon Regulatory Factor 3 On The Immune Response To Herpes Simplex Virus Type I Infection

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THE IMPACT OF INTERFERON REGULATORY FACTOR 3 ON THE IMMUNE RESPONSE TO HERPES SIMPLEX VIRUS TYPE I INFECTION

By

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Table of Contents

List of Figures

List of Tables

ABSTRACT OF THE DISSERTATION

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The type I interferon (IFN) cascade is critical in control of herpes simplex virus type I (HSV-1) infection and relies on specific recognition molecules to rapidly signal viral infection via interferon regulatory factor-3 (IRF-3) -dependent pathways. The absence of these recognition molecules or the loss of IRF-3 would be predicted to render early recognition pathways inoperative and thus impact viral infection. However, previous results had produced contradictory results in terms of the role of IRF-3 during HSV-1 infection. In this study, infected IRF-3$^{-/-}$ immune cells were found to support increased HSV-1 replication compared to control cells. In addition, IRF-3 deficient cells exhibited delayed type I IFN synthesis following infection and were partially restored in the presence of exogenous IFN; blockade of the type I IFN receptor resulted in similar titers in control and IRF-3$^{-/-}$ cells. Together, the data demonstrated that defective and deficient type I IFN production in IRF-3$^{-/-}$ cells resulted in increased HSV-1 replication *in vitro*. *In vivo*, IRF-3 deficiency was found to have no significant impact on HSV-1 replication in peripheral tissues following ocular challenge with a laboratory (17) or a

neurovirulent strain (McKrae) of virus. However, IRF-3$^{-/-}$ mice were significantly more susceptible to central nervous system infection following both peripheral and intracranial infection with HSV-1. Increased viral replication and inflammatory cytokine production were observed in brain tissues of IRF-3$^{-/-}$ mice compared to control mice. In addition, the production of IFNβ and IFNα was delayed and reduced in IRF-3$^{-/-}$ brains. These data demonstrate a critical role for IRF-3 in control of central nervous system infection following HSV-1 challenge. Together, the data illustrate the importance of IRF-3 mediated pathways in initiating the type I IFN cascade necessary to control HSV-1 infection both *in vitro* and *in vivo*.

Chapter I

Introduction

The Herpes Virus Family

Herpesviridae constitutes a family of ubiquitous viruses that infect a variety of hosts ranging from fish to mammals (122). The nearly 130 identified members of the herpesvirus family are divided into three subfamilies on the basis of biological properties: *Alpha-, Beta-,* and *Gamma-* herpesviruses (165). *Alphaherpesviruses,* which include herpes simplex virus type 1 (HSV-1) and herpes simplex type 2 (HSV-2), have a variable host range, short reproductive cycle, rapid spread, and lyse host cells *in vitro* and *in vivo*. In contrast, *betaherpesviruses*, which include human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV) , have a restricted host range, a long life cycle, and grow slowly in culture. Finally, *gammaherpesviruses* are restricted to the family or order of their natural host and include Epstein Barr Virus (EBV); the members of this group effectively replicate within immune cells especially T and B lymphocytes (165). Despite initial classification based mainly on biological properties, nucleic acid and protein sequence similarities have since validated grouping into these functional families. However, each member of the *herpesviridae* family shares several significant properties.

The hallmark of the herpes virus family is the ability to establish latency and a lifelong infection. Latency is characterized by limited viral gene expression, stable maintenance of the viral genome, and the absence of infectious virions (165). While the stimuli remain unknown, herpesviruses in the latent state are periodically induced to reactivate causing recurrent infection in the host. The absence and reemergence of infectious virus distinguish latency from chronic or abortive infections respectively. Establishment of latency in varying cell types also distinguishes between the three herpesvirus families. *Alphaherpesviruses* establish latency within sensory ganglia;

betaherpesviruses establish latent infection in secretory glands, lymphoreticular cells, the kidney, and other tissues. Finally, *gammaherpeseviruses* frequently establish latency in lymphoid tissues, specifically B and T-cells.

Despite the wide host ranges, each member of the herpesvirus family also maintains a similar structure with four distinct components: core, capsid, tegument, and envelope (165). The inner core contains the linear double-stranded DNA genome which is surrounded by an icosohedral capsid made up of viral proteins. The lipid envelope makes up the outer most layer of the virion and is composed primarily of host cellular membrane and viral glycoproteins. These viral glycoproteins are non-randomly distributed throughout the envelope and form the spikes associated with herpes virions. Finally, the tegument separates the envelope from the capsid; the amorphous tegument is made up of several viral proteins that function in the host cytoplasm, nucleus, or both. Many of these proteins are dispensable for viral replication, but appear critical for virulence. Together, these components form herpes virions ranging in size from 120 to nearly 300nm with tegument thickness and envelope composition contributing to the variability in size.

In addition to similar structure, the herpesvirus family shares several common traits during infection. Each utilizes glycoproteins within the envelope to mediate binding, attachment, and fusion to the host cell. Upon entry, herpesviruses traffic their genome and other components to the nucleus where viral transcription, DNA replication, capsid formation, and packaging of viral DNA occur. Utilizing its large double-stranded DNA genome, herpesviruses also encode a wide array of proteins involved in nucleic acid metabolism, DNA synthesis, protein processing, and immune antagonism.

Following DNA packaging, capsids acquire and lose a primary envelope composed of the nuclear membrane. Finally, the tegument and envelope are acquired in the cytoplasm and the progeny virions are prepared to exit the cell. Production of infectious herpesvirus progeny, in contrast to latency, results in destruction of the infected host cell.

Herpes Simplex Virus Type I

The thesis work described herein focuses on infection with herpes simplex virus type I (HSV-1), a member of the *alphaherpesviridae* family (165). HSV-1 is a widespread human pathogen that has high seroprevalence in adults (222). Among the first human herpes viruses to be discovered, HSV-1 infection has served as a research model in numerous areas including the nervous system, membrane structure, and gene regulation, in addition to infectious disease (164). HSV-1 infection also causes numerous diseases in humans and is the target of on-going vaccine research (96).

Structure

Like all herpesviruses, HSV-1 is composed of the four major structural components: genomic core, capsid, tegument, and envelope. The virus has a large double-stranded DNA genome comprised of nearly 150 kilo-base pair divided into a unique long and unique short region (121, 163). Enclosed within the viral capsid, the genome encodes as many as 84 unique genes involved in the viral life cycle (164). In addition to the capsid, the viral envelope, composed of viral glycoproteins and host cell membrane, encases the HSV-1 tegument. The tegument, which constitutes the layer between the envelope and capsid, is released into the cytoplasm shortly after infection (132). The

tegument is among the best-characterized and most studied groups of viral proteins and is critically involved in HSV-1 life cycle.

Viral Replication

HSV-1 replication is divided into several major events. Infection is initiated via attachment of viral glycoprotein C and B (gC and gB) to the cell surface, glycoprotein D binding of cell-surface co-receptors, and fusion of the viral envelope and plasma membrane(154, 164, 188-189, 208). Following fusion, the capsid is transported to the nucleus and the tegument proteins are released into the cytoplasm, accompany the DNA into the nucleus, or remain associated with the capsid (164, 205). In the nucleus, viral transactivators, in combination with the host machinery, initiate ordered transcription of viral genes divided into four kinetic classes (213). The α genes, the immediate early kinetic class, consist of five viral proteins that prime the transition from cellular to viral gene expression (164). The β genes, the early kinetic class, follow the α genes with expression of viral proteins involved in replication of viral DNA. The γ1 genes, the leaky late genes, can actually be expressed prior to DNA replication, but their maximal expression is DNA replication-dependent. These γ1 genes include structural proteins, glycoproteins, and immune antagonists. Finally, the γ2 genes, the true late structural genes, are expressed and are dependent on viral DNA synthesis for expression.

Following γ1 gene expression, synthesis of progeny virions is initiated. The capsid protein, a γ1 gene, localizes to the nucleus for insertion of the viral DNA (131, 164). The complete nucleocapsid then egresses through the nuclear membrane and eventually into the golgi apparatus acquiring tegument proteins during the process.

Within the golgi apparatus, final maturation of the viral glycoprotein occurs and the virion is ready to infect a new cell either through release through the plasma membrane or through direct cell-to-cell contact (164, 207). Productive HSV-1 infection results in death of the host cell due to a variety of cellular responses to infection including changes in host chromatin, alteration of cell membranes, and formation of intranuclear inclusion bodies (164).

Life cycle and Latency

As mentioned earlier, the ability to establish a lifelong latent infection is the hallmark of herpesvirus infections (165). During HSV-1 infection, the virus life cycle begins with lytic replication in peripheral mucosal epithelia (164). The virus is then able to enter the nerve termini and is transported through the axon in a retrograde fashion to the sensory ganglia (105). Upon reaching the ganglia, the virus can continue a lytic infection in neurons. Following entry into the CNS during acute primary infection HSV can cause life-threatening encephalitis; however, the virus usually shifts to a quiescent latent state in the peripheral nervous system,, in sensory ganglia. The latent state is characterized by an episomal viral genome, the production of latency-associated transcripts (LATs), and the lack of infectious progeny virions (165). The host immune system also contributes to the establishment of latency by suppressing infection and limiting spread (13, 204). Inflammation and CD8+ T-cells have also been implicated in maintaining HSV-1 in a latent state (37-38). Periodically, the latent HSV-1 is induced to reactivate via "stress" stimuli including immune suppression, physical/emotional stress, or exposure to ultraviolet (UV) light (164). Reactivated viral particles are then

transported in an anterograde direction from the sensory neuron to the primary site of infection resulting in asymptomatic shedding or recurrent infectious lesions at the original infection site (105).

Clinical manifestation

HSV-1 infection is wide spread in the human population reaching 70-80% prevalence in the adult population (222). The host first encounters the virus via contact with secretions, skin, or mucosal membranes from symptomatic or asymptomatic individuals shedding virus (218). Oral infection often results in the most frequent manifestation of HSV-1 infection: herpes simplex labialis, the common cold sore (46). Following primary oral infection, HSV-1 establishes latency in the trigeminal ganglia; periodic "stress" stimuli result in reactivation and result in HSV-1 induced cold sores at the initial site of infection in 20-40% of HSV-1 seropositive individuals (8). In immune competent individuals, recurrent infection is less severe than primary infection and is controlled within 1-10 days after initial onset of symptoms (152). HSV-1 is also associated with genital herpes, herpetic stomatitis in the mouth, and cutaneous herpetic infections like herpetic whitlow, eczema herpeticum, and herpes gladiatorum(8) . In general, these infections are resolved in immune competent host and cause minimal long term damage.

In contrast, HSV-1 infection may lead to serious infection of the eye and the CNS. Infection of ocular tissue resulting in serious eye disease can happen via direct entry or spread from non-ocular sites like the mouth (90). Ocular HSV-1 infection often initiates severe immune reactions inducing blepharitis, conjunctivitis, retinitis, iridocyclitis,

epithelial and stromal keratitis (8, 206). Herpetic stromal keratitis (HSK), a condition that results in stromal damage, scarring, and loss of vision, is among the leading causes of infectious blindness in developed countries (152). In addition to ocular disease, HSV-1 remains among the most common causes of viral encephalitis (92-93). Herpes simplex encephalitis (HSE) is a rare, but life threatening consequence of infection of the nervous system (209). Through recurrent infection in adults or maternal transmission to neonates, HSV-1 infects the brain and causes acute inflammation and significant pathological damage (92-93, 217). If untreated, HSE mediated damage leads to nearly 70% lethality and few patients return to normal function (209).

Diagnosis, treatments, and vaccines

Diagnosis of HSV-1 infection is typically based on clinical histology and presenting features, although various tests have been developed to confirm HSV-1 infection including changes in cytology, plaque assay, and serological methods (8). However, detection of viral DNA by PCR is generally considered to be the most sensitive measure for presence of HSV-1 (22). Therapy for HSV-1 infection ranges from no treatment to a combination of antiviral drugs and corticosteroids (78, 179). The most commonly utilized drug against HSV-1 is acyclovir, a nucleoside analogue (8). Acyclovir is converted to its active form by viral thymidine kinase and incorporated into viral DNA acting as a chain terminator and inhibiting viral replication (220). Acyclovir also targets the viral DNA polymerase (ref). It can be given topically, orally, and intravenously permitting it to treat a variety of HSV-1 infections including ocular, genital, or HSE (8). Additional treatment utilizing corticosteroids to dampen the immune

response to viral infection have also been explored, but the results remain unclear on its efficacy or side-effects (78).

Research continues to develop new treatments for current HSV-1 infections and preventative measures to impede future infection. While the majority of HSV-1 drugs are nucleotide analogues that interfere with viral replication, several new categories of antiviral drugs are being examined including helicase inhibitors, TLR agonist, and therapeutic vaccines (220). In addition, work continues on development of a vaccine that confers protection against HSV-1 infection. Both subunit based and live-attenuated HSV-1 vaccines have been examined for efficacy in animal models and clinical trials (96-97). Yet, none to date have been effective in protection (95, 220). Several factors contribute to the difficulty in developing a HSV-1 vaccine, most notably opposition to the use of a live-attenuated vaccine, and a failure of subunit vaccines to establish robust local mucosal immunity (220).

Animal models

For HSV-1 research, a variety of animal models have been studied that mimic human disease including rabbits and guinea pigs (72, 107, 151, 203, 218). However, the mouse model of HSV-1 infection remains among the most common and practiced methods for *in vivo* research (151). The outcome of HSV-1 infection in mice is highly dependent on a variety of factors including virus and mouse strain, competence of the immune system, and route of infection. For example, certain virus strains such as HSV-1 strain McKrae are more virulent in mice than other HSV-1 strains, though the exact mechanism for the change in virulence is unclear (153). In addition, mouse strains like

C57BL/6 are more resistant to HSV-1 challenge than Balb/C mice; these difference in strains have been attributed to changes in the innate immune response (115). Mice deficient in immune pathways have also been demonstrated to have increased susceptibility or alternatively, enhanced resistance to viral infection (108, 116, 144). In addition, the route of infection plays a major role in determining the pathogenesis of HSV-1 *in vivo*. Together, these factors permit evaluation of several elements of HSV-1 infection. In these studies, two routes of *in vivo* HSV-1 infection are utilized: ocular infection via corneal scarification and direct intracranial inoculation.

The ocular route of infection provides a physiologically relevant model for HSV-1 infection *in vivo* (107). To mimic human disease, mouse eyes are scarified to permit viral infection of the corneal stroma; stromal infection results in lytic infection at the primary site of infection and HSV-1 can then enter the nerve termini that innervate that region of the cornea (105). The virus is then transported in a retrograde manner, through the axon to the trigeminal ganglia; HSV-1 replication in the trigeminal ganglia can be measured as early as day 1 and peaks between day 3-6 post infection (218). HSV-1 can continue to travel to the brain in a retrograde spread, or travel in an anterograde direction leading to infection of the periocular skin, a measure of zosterform spread from the trigeminal ganglia back to new peripheral tissues (14, 193). Viral titers in the cornea are measured via eye swab while the trigeminal ganglia, brain, and periocular skin can all be measured via plaque assay of harvested tissue homogenates.

In addition to examination of primary disease, the cornea model of infection also permits evaluation of latency (218). The two primary measures of latency are establishment and reactivation. Establishment refers to the presence of the HSV-1

genome in the trigeminal ganglia in the absence of viral replication; by 28 days post ocular infection, HSV-1 replication is no longer detectable and establishment is evaluated by PCR for viral DNA (23, 201). Reactivation refers to the ability of the virus to shift from latency to lytic infection and is typically measured by peripheral shedding of virus. *In vivo*, mice have a very low rate of spontaneous reactivation compared to humans or rabbits (141, 218); however, exposure to UV light *in vivo* or ex-vivo transplantation of trigeminal ganglia permits measurement of viral reactivation (107, 184). In recent years, examination of in situ hybridization in the trigeminal ganglia of latently infected mice has revealed "rare neurons" that express high levels of lytic HSV-1 transcripts, viral DNA, and protein (47). This low level of lytic antigen production absent infectious virus has been termed molecular reactivation and likely contributes to the generation of immunity during latency (47, 52, 94).

Both the ocular and the intracranial injection models evaluate the ability of HSV-1 to invade and replicate within the CNS (42). Cornea infection results in trafficking of the virus from the periphery into the brain and permits examination of neuroinvasiveness (105). HSV-1 replication in the brain also provides a measure of viral fitness in the CNS; however, several factors impact neuroinvasiveness including replication kinetics in peripheral tissues, ability to be efficiently retrogradely transported, and actions of the immune system. Neuroinvasiveness is highly virus and mouse strain dependent(12, 42, 117). To control for these external factors in peripheral tissues, HSV-1 infection via direct intracranial inoculation permits evaluation of the virus' ability to replicate within the brain tissues (42). This CNS model of infection mimics human HSE and results in high morbidity and mortality rates *in vivo*. Together, the ocular and intracranial

inoculation routes provide *in vivo* mouse models for two important HSV-1 human diseases.

The Immune Response to Virus

In response to viral infection, interferons induce signaling cascades that arm immune defenses and provide the initial line of defense against invading pathogens and malignant cells (77). The term interferon was originally derived from the ability of these secreted factor to "interfere" with viral replication in host cells (77, 169). Divided into type I and II categories, interferons initiate transcription of hundreds of genes through binding their individual receptors (84, 155). The products of these interferon stimulated genes have anti-viral, immune-modulatory, and cell regulatory functions which are critical in the control of viral infection *in vitro* and *in vivo*(56, 84, 169). In the absence of either the type I or type II interferon cascades, the host fails to mount effective immune responses and becomes very susceptible to pathogen and tumor challenge (21, 73, 108, 129, 210). Therefore, interferons play a critical role in initiating the immune response to viral challenge.

Type I interferon, also known as viral IFN, can be produced by nearly every cell type within the host (169). Located together on chromosome 9 in humans or chromosome 4 in mice, type I IFN genes are divided into several categories including IFNβ, IFNα, IFN-w and IFN-t (155, 177). While the need for multiple types remains unclear, each form of type I IFN shares structural homology that permits binding to the single, common type I IFN receptor (155). The type I IFN receptor is composed of two subunits, IFNAR1 & 2, which are associated with Janus activated kinase (JAK) 1 and tyrosine kinase (TYK) 2 (34, 155). Upon binding of type I IFN, the IFNAR stimulates

auto-activation of JAK1 and TYK2 resulting in tyrosine phosphorylation of STAT1 and STAT2. The phosphorylated STAT1 and STAT2 form heterodimers and in conjunction with interferon regulatory factor nine (IRF9), become the IFN-stimulated gene factor 3 (ISGF- 3)(34, 155). This complex travels to the nucleus and binds IFN-stimulated response elements (IRSE) in the DNA and initiate the type I IFN cascade.

In contrast to type I, type II IFN consists of a single form, IFNγ. Structurally distinct from the type I IFNs, IFNγ is produced mainly by T-cells and natural killer cells and targets immune cells including macrophages (77, 155, 169, 177). Upon binding IFNγ, the two subunits of the type II receptors, IFNG1 and IFNG2, induce the phosphorylation of STAT1 via JAK 1 and JAK 2 (155). The phosphorylated STAT1 forms a homodimer and translocates to the nucleus, binding to IFNγ activated sites (GAS) elements upstream of target ISGs. This leads to the transcription of numerous target genes and various outcomes that encompass the type II IFN cascade (77, 84, 169).

While both type I and type II IFN have been shown to be necessary in the control of viral replication *in vitro* and *in vivo* (21, 73, 108, 129, 210), the recognition pathways of the immune system, the focus of this thesis, primarily produce type I IFN in response to viral challenge. Therefore, herein, the focus will be on the type I IFN system with implicit acknowledgement of the importance of type II IFN in control of viral infection.

Interferon Stimulated Genes and Down Stream Effects.

While type I interferon has no reported enzymatic activity, the hundreds of induced interferon stimulated genes (ISGs) mediate a variety of biological responses that impact the ability of the virus to function and can elicit an anti-viral state within the cell

(84, 155). Among the most prominent anti-viral ISGs is the dsRNA-activated serine/threonine protein kinase, PKR. Upon binding double-stranded RNA, PKR is activated and phosphorylates eIF2α leading to inhibition of protein translation (51, 169). Several antiviral ISGs target other parts of the virus replication cycle, for example, 2'5' oligoadenylate synthetase and RNase L target viral RNAs for degradation (84, 162). The myxovirus-resistance proteins (Mx), IFN-inducible GTPases, were among the first studied ISGs and have broad functions in both the nucleus and cytoplasm on multiple types of viruses (77, 166, 169). ISG15, originally identified as a ubiquitin homologue, protects against viral mediated degradation of immune components or modifies enzymatic function to enhance the antiviral state (166). Yet, only a minor population of ISGs have been fully evaluated *in vitro* or *in vivo*.

Type I IFN signaling is thought to induce or modify nearly 2000 genes. While well over 300 have been identified to be highly induced via microarray studies, the majority of these ISGs have been categorized as having no "direct" antiviral activity; instead, they encompass a wide variety of functions that contribute to the antiviral state (36). Type I IFN signaling has been shown to have immuno-modulatory and cell regulatory functions (84, 169). Studies have demonstrated an increase in expression of MHC class I and II, necessary components for antigen presentation to T-cells (36, 77, 166). Similarly, several ISGs encode chemokines and adhesion molecules critical for the trafficking of lymphocytes to the area of infection (36). IFN signaling also leads to increased expression of signaling molecules and transcription factors often involved in the inflammatory response (36, 155). IFN has been shown to upregulate genes involved in both protein degradation as well as apoptosis (10, 36). Numerous other ISGs have yet

to even be categorized and fully evaluated. Together, in combination with the known antiviral ISGs, IFN mediated effects result in host cells being rendered inhospitable to virus infection.

IFN, ISG, and HSV-1 antagonism

Naturally, viruses have developed mechanisms to interfere with type I IFN and its downstream effects (55, 84, 177). At any step within the type I IFN cascade, viruses can block, sequester, or inhibit elements of the IFN signaling machinery. In addition, successful viruses often antagonize the production or function of ISGs, most notably PKR and RNaseL (84). HSV-1 is among the most adept viruses at subverting the immune response; it encodes several viral proteins that delay and interfere with the type I IFN cascade through known, and yet to be determined, mechanisms.

Among the best characterized mechanisms of viral antagonism of immunity is HSV-1 neutralization of the PKR pathway. HSV-1 encodes a protein, ICP34.5,which has been implicated in a variety of functions including inhibiting autophagy, processing glycoproteins, and facilitating nuclear and cytoplasmic egress (16, 79, 146). Its most prominent function, however, has been associated with interfering with host-protein shutoff . ICP34.5 recruits protein phophatase 1a (PP1α) and directs it to dephosphorylate eIF-2α, the target of activated PKR (60-61). The reduction in phosphorylated eIF-2α removes the PKR induced block and permits viral protein synthesis to continue uninterrupted. In the absence of ICP34.5, HSV-1 is highly neuroattenuated *in vivo* with restoration occurring only in PKR-/- mice (110). In addition to ICP34.5, HSV-1 encodes a second PKR antagonist, US11. US11 acts by binding directly to PKR and preventing

activation by dsRNA or PACT (40). A suppressor mutant lacking ICP34.5 had partial restoration if US11 was under control of an immediate early promoter (25-26, 215). Together, these data demonstrate that multiple HSV-1 proteins target PKR and downstream pathways that contribute to the anti-viral state.

In addition to ICP34.5 and US11, the virus encodes several other known immune antagonists. ICP0, an immediate early transactivator of HSV-1, interferes with several aspects of the type I IFN cascade including STAT-1 signaling, with induction and function of ISGs, and resistance to type I IFN signaling (43, 59, 135, 137-138, 187). The virion host-shut off protein (vhs), a viral riboendonuclease, has also been implicated in antagonizing the ISG antiviral response by degrading host transcripts, targeting type I IFN receptors, and interfering with the activity of JAK/STAT pathway through activation of the suppressor of cytokine signaling 3 (SOCS-3) (29, 226-227). In the absence of either ICP0 or vhs, HSV-1 becomes very sensitive to type I IFN and the virus is attenuated both *in vitro* and *in vivo* (113, 137). However, mutants lacking ICP0 or vhs are partially restored in hosts lacking type I IFN signaling implying their action the type I IFN cascade (108, 148, 150). In more recent work, ICP27, an immediate early viral protein, has been identified in interfering with JAK/STAT signaling and the activity of ISG15 (80-81).

These five viral proteins (ICP34.5, US11, ICP0, vhs, and ICP27) represent the best characterized immune antagonists encoded by HSV-1. Yet, other viral proteins have also been implicated in influencing the antiviral state including ICP47 and UL13 (19, 145, 183, 227). In addition, the virus has been found to counter several antiviral processes including RNaseL and 2’5’ oligoadenylate activity, although no conclusive

mechanism has been established. Together, the data demonstrate that HSV-1 employs significant genetic capital in neutralizing type I IFN and its downstream cascade.

Pathogen Recognition Pathways

While type I IFN and its downstream effects have been examined for several decades, recent research has focused on the pathways that induce the type I IFN cascade (18, 67, 178). Over the past few years, the discovery of pattern-recognition receptors (PRRs), adaptors, and signaling molecules has provided detailed mechanisms for the recognition of a variety of invading pathogens. The host immune system relies on these early recognition pathways to identify infection and induce the production of type I IFN, specifically IFNβ. In the past few years, the area of pattern recognition has been divided into two branches: the extracellular recognition pathway comprised of the toll-like receptor family (TLR) and the intracellular recognition pathway represented by the Rig-I like receptors (RLR) (18). Each family provides surveillance for specific areas within the host cell environment and utilizes adaptors and signaling molecules to drive production of IFNβ following infection.

Toll-Like Receptor Signaling

The toll-like receptor (TLR) family consists of several transmembrane receptors that recognize pathogen-associated molecular patterns (PAMPs) (3). TLRs sample extracellular contents surrounding host cells either through expression on the cell surface or within endosomes. Originally discovered in Drosophila, 10 TLR genes are expressed in mice and humans; each recognizes distinct PAMPs associated with bacteria, viruses, or

fungi (3, 77, 86, 111). Together, these receptors play a key role in activating immune cells inducing phagocytosis, cytokine secretion, and enhanced antigen presentation in response to infection (77).

The majority of the TLRs most commonly associated with viral recognition bind nucleic acid motifs within the endosome. TLR3 was among the first to be characterized binding poly IC and double-stranded RNA (4). In a similar fashion, TLR9 was found to recognize unmethylated DNA and both TLR7 and 8 bind to single-stranded RNA elements (62-63). In addition to recognition via nucleic acid motifs, viruses have also been described to activate TLR2 and TLR4 through recognition of viral proteins (65, 104, 235). Together, the data demonstrate that multiple TLR pathways are involved in the recognition of viral pathogens.

With the exception of TLR3, each of the TLRs transmits its downstream signals via myeloid differentiation primary response gene 88 (MYD88) (199). This adaptor is recruited via its C-terminal Toll-IL-1 receptor (TIR) domain which interact with the TIR domains found within the TLRs(20, 123). Upon stimulation, MYD88 recruits IL-1R associated kinase (IRAK) via its N-terminal death domain (216); IRAK is then activated by phosphorylation and in association with tumor necrosis factor receptor-associated factor 6 (TRAF6), which leads to activation of JNK and NFκB pathways (20, 139). MYD88 activation of IRF pathways has also been linked directly through TRAF6 (68, 87). In the absence of MYD88, the immune response to double stranded RNA, unmethylated DNA, and single stranded RNA motifs are ablated demonstrating the critical role for MYD88 in TLR signaling (4, 58, 62).

The MYD88-independent pathway relies on the TIR domain containing adaptor-inducing IFN (TRIF) for downstream signaling(175). In both TLR3 and TLR4 signaling, TRIF acts as an adaptor facilitating activation of TBK-1 and subsequent activation of IRF-3. While TLR3 exclusively uses the MYD88-independent pathway, TLR4 utilizes both MYD88 dependent and independent pathways to stimulate the immune response to infection (3, 66). Upon binding LPS or a viral ligand, TLR4 stimulates both MYD88 through interaction with MAL and TRIF through association with the TRIF related adaptor molecule (TRAM) to activate pathways leading to NFκB and IRF-3 activation (3, 86, 199). Similarly, upon binding double stranded RNA, TLR3 activates TRIF pathways leading to downstream signaling (175). In the absence of TRIF, the induction of IFNβ in response to dsRNA or LPS is severely impaired, demonstrating the importance of TRIF in inducing MYD88-independent response to virus infection (64).

The TLR family has been implicated in the recognition and immune response to numerous viruses including HSV-1 both *in vivo* and *in vitro*. In the absence of TLR9, plasmacytoid dendritic cells (pDCs) fail to induce type I IFN production in response to HSV-1 challenge (100). HSV-1 glycoproteins have been identified in the activation of the TLR2 pathway (7, 171). TLR3 has been shown to be important for protection against herpes simplex encephalitis (HSE) in humans (24, 231). Yet, in contrast to other viruses that encode multiple pathways to interfere with the TLR pathways, very few HSV-1 processes have been identified to target TLRs. ICP0 has been implicated in antagonizing TLR signaling through its interaction with USP7 (35). However, the absence of TLR or MYD88 pathways has had minimal impact on HSV-1 replication; in fact, the absence of several TLR pathways often resulted in reduced pathology *in vivo*

(100, 102-103). Together, these results suggest that HSV-1 may be recognized via TLR pathways, but it has minimal impact on infection. Thus, HSV-1 has devoted minimal genetic capital to control this pathway.

Intracellular viral recognition pathways

Similar to the TLRs, the intracellular pathways of virus recognition utilize pattern recognition receptors to recognize PAMPs within the cytoplasm. The Rig-I like receptor family, comprised of retinoic acid inducible gene (RIG-I) and melanoma differentiation associated gene 5 (MDA5) recognize elements of viral RNA within the cytosol (18, 54, 229). The closely related MDA-5 and RIG-I proteins contain two similar domains: A N-terminal caspase recruitment domain (CARD) and a C-terminal DExD/H box RNA helicase (66). The C-terminal helicase has been demonstrated to confer RNA binding, while the CARD domain is required for interaction with downstream adaptors (66). Despite their similar domains, MDA-5 and RIG-I bind distinct RNA motifs resulting in recognition of different subsets of viruses. MDA-5 binds longer molecules of viral double stranded RNA and is required for recognition of positive-sense singles including norovirus and picornaviruses (54, 120, 195) . In contrast, RIG-I detects single stranded RNA sequences with a free 5' triphosphate or short fragments of double stranded RNA, each typically associated with negative-stranded RNA viruses (18, 83, 168). In the absence of either RIG-I or MDA-5, mice are defective for the induction of type I IFN in response to specific viral pathogens. These results demonstrate the critical role for the RLR family in responding to viral challenge.

In addition to the RLR family, additional intracellular pathways have been implicated in the recognition of microbial DNA (18). While the pathways have not yet been elucidated, several studies have demonstrated an IFN response to intracellular DNA targets (74, 143, 192). The discovery of a DNA receptor, DNA-dependent activator of IRFs (DAI), suggested a potential mechanism by which DNA recognition occurs (198). However, further studies minimize the impact of DAI and suggest additional DNA sensors are involved in the immune response (176, 214). In contrast, another study suggests that DNA recognition is mediated through RNA polymerase III conversion of DNA to RNA; the newly formed RNA then activates RIG-I driving production of type I IFN (1). Together, these data provide sufficient evidence to conclude that a DNA sensing pathway exists and contributes to the induction of type I IFN in response to virus infection.

Similar to the TLR pathways, the intracellular pathways are dependent on an assortment of adaptors and signaling molecules. The primary adaptor for the RLR family was independently identified by different four groups and given the following names: IFNB promoter stimulator 1 (IPS-1), mitochondrial antiviral signaling (MAVS), CARD adaptor inducing IFNB (Cardif), and virus induced signaling adaptor (VISA) (88, 133, 181, 224). For the purpose of simplicity, this molecule will be referred to as IPS-1. IPS-1 contains an N-terminal CARD domain mediating interaction with the RIG-I and MDA-5 (88). In addition, IPS-1 contains a C-terminal transmembrane domain that targets itself to the outer mitochondrial membrane (181). Upon binding target RNA, RIG-I and MDA-5 engage IPS1 resulting in recruitment of several signaling molecules including TRAF2, TRAF6, Fas-associated protein with death domain (FADD), and

receptor interacting protein 1 (RIP-1)(88, 224). These signaling molecules activate TBK and in turn, lead to the activation of NFκB and IRF pathways, driving production of type I IFN. In the absence of IPS-1, the host has a severe defect in RIG-I and MDA-5 signaling and is susceptible to a variety of RNA virus infections(101).

A second adaptor identified as part of the intracellular recognition machinery is the stimulator of interferon genes (STING). STING, an endoplasmic reticulum transmembrane protein, has been shown to interact with both IPS1 and RIG-I inducing production of type I IFN (75, 194, 234). Further examination revealed that STING is necessary for type I IFN production in response to intracellular DNA (76). In the absence of STING, IFNβ production was ablated following challenge with non-CpG DNA; STING also contributed to host defense against both RNA and DNA pathogens. Together, the data demonstrate a critical role for both STING and IPS-1 in the intracellular pathways leading to type I IFN production.

Despite their relatively recent discovery , many studies have revealed a variety of viruses that target these receptors and adaptors (18). Through inhibition, cleavage, and degradation, viral proteins interfere with the activity of the intracellular recognition pathway and delay and inhibit type I IFN production (18, 50, 178). To date, no HSV-1 proteins have been directly implicated in antagonizing the RLR or DNA sensing pathways. In fact, the mechanism for HSV-1 recognition is still not fully understood. Several studies have demonstrated that HSV-1 induces a MYD88 independent production of type I IFN (31, 158). Additional studies have implicated RIG-I, and STING as possible components involved in identification(75, 160) . Yet, questions remain on the exact pathways induced to produce type I IFN following HSV-1 infection.

Initiation of type I IFN production

IFNβ is among the first type I IFN molecules produced and is critical in the type I IFN signaling cascade (45, 202). Rapidly induced via pathogen recognition pathways, IFNβ acts as an autocrine and paracrine activator of the type I IFN receptor, leading to amplification of the type I IFN response (156). In the absence of IFNβ, IFNα subspecies fail to compensate, resulting in increased host susceptibility to viral challenge (39, 169). Together, the data demonstrate the importance of IFNβ to the immune response to viral infection.

Regulation of IFNβ production occurs at the gene transcriptional level by nucleosome obstruction of the transcriptional start site (2, 66, 114). Upon viral infection, pathogen recognition pathways induce activation of factors that bind to IFNβ promoter regions and facilitate nucleosome displacement from the IFNβ transcriptional start site (114). The IFNβ promoter region contains four positive regulatory domains (PRDs): I, II, III, and IV (66, 142, 147, 178). The PRDs bind to transcription factors that are either present constitutively or induced by signaling via the type I IFN receptor. PRD I and III bind members of the interferon regulatory factor (IRF) family, notably IRF-3 and IRF-7 (91). In contrast, PRD II and IV bind nuclear factor kB (NFκB) and AP-1 (a heterodimer of activating transcription factor 2 with c-JUN). Following viral recognition, the activated IRFs, NFκB, and AP-1 bind to the PRDs on the IFNβ promoter and recruit the high mobility group protein, HMG-1 forming the IFN "enhancesome" (91). The enhancesome then recruits histone acetylation transferases (HATs) to aceylate lysine residues of histone H3 and H4 in the nucleosome. Meanwhile, RNA polymerase II is

recruited to the promoter and aceylation of the histone results in recruitment of a nucleosome modification complex which displaces the nucleosome (2, 66, 114). Displacement permits recruitment of TFIID to the IFNβ promoter and induction of IFNB follows(2). Following production, IFNβ then acts in an autocrine and paracrine manner initiating the type I IFN cascade in the infected as well as bystander cells (177). As the infection continues, the levels of IFNβ plateau as other forms of type I IFN are produced by infiltrating immune cells, most notably variants of IFNα by pDCs (9, 27).

IRF-3 & IRF-7

In recent years, interferon regulatory factor 3 and 7 have been identified as key components of the early recognition response leading to the type I IFN cascade (18, 66-67). IRF-3 is constitutively expressed and located in the cytoplasm of host cells in an inactive form (66). Following stimulation via pathogen recognition pathways, the transcription factor undergoes phosphorylation at a serine residue within its C-terminal region (134, 180). Phosphorylation induces dimerization and formation of a homodimer, or a heterodimer with activated IRF-7 (196). The dimer is then transported to the nucleus where it binds co-activators CBP and p300 (112). This complex then targets the IFNβ promoter at the PRD I and III sites and facilitates production of IFNβ (2).

In addition, several gene groups have been identified as partially or totally dependent on IRF-3 for activation; these genes fall into two categories: genes dependent on IRF-3 binding for expression and genes augmented by IRF-3 in the context of type I IFN signaling (6, 44, 57). The IRF-3 augmented group includes several genes primarily associated with the immune and interferon response including Rantes, ISG15, and ISG

60 (6). In contrast, the IRF-3 dependent group includes genes from a variety of categories including cell stress, apoptosis and proliferation, in addition to immune response genes (6, 44). A subset of type I IFN, including IFNβ, IFNA4 and IFNa5, are among the most notable IRF-3 dependent genes identified. In the absence of IRF-3, the production of IFNβ is severely attenuated both *in vitro* and *in vivo* (67, 69). Together, these data demonstrate the importance of IRF-3 in IFNβ production and initiation of the type I IFN cascade.

In contrast to IRF-3, IRF-7 activity is highly dependent on type I IFN signaling. IRF-7 is expressed at very low basal levels and has a short half life within the cell (174, 202). Upon type I IFN signaling, IRF-7 transcription is strongly induced via the ISGF3 transcriptional activating complex (173, 202). In an inactive form, IRF-7 resides in the cytoplasm and is phosphorylated on its C-terminal regions upon stimulation by viral infection (66). IRF-7 then forms a homodimer or heterodimer with IRF-3 and translocates to the nucleus and activates production of additional type I IFN, primarily subtypes of IFNα (173). Together, the data demonstrate a critical role for IRF-7 in the type I IFN positive feedback look.

These findings led to a three step model of type I IFN induction (136, 174). First, during the sensitization phase, viral infection is detected via pathogen recognition pathways, leading to IRF-3 phosphorylation and dimerization (18). Activated IRF-3 translocates to the nucleus, forms the enchancesome, and drives expression of IFNβ (91, 136, 230). The released IFNβ initiates the second stage of the type I IFN response, the inductive phase. IFNβ acts on both self and bystander cells leading to the expression of IRF-7 via signaling through the type I IFN receptor (173). Viral activity continues to

stimulate pathogen recognition pathways leading to IRF-7 activation. In turn, IRF-7 leads to further production of type I IFN and provides a positive feedback loop that drives the type I IFN cascade. The continued type I IFN production initiates the final phase of the IFN response, amplification. With production of numerous subset of type I IFN and additional IFNAR receptors, the cell amplifies interferon stimulated genes resulting in induction of a full-antiviral state (84, 169). Thus, IRF-3 was thought to govern the initial induction of type I IFN and IRF-7 critical to subsequent stages.

However, *in vitro* and *in vivo* studies suggested a less IRF-3 dependent model for type I IFN induction (69). Utilizing deficient mouse embryonic fibroblasts (MEFs), IFNβ message was severely decreased in both IRF-3-/- and IRF-7-/- MEFs. In contrast, IFNα message was normal in IRF-3-/- MEFs, but ablated IRF-7-/- MEFs following challenge with EMCV, VSV, and HSV-1. In either case, the loss of these components had no impact on endpoint viral replication (69). *In vivo*, intravenous challenge with HSV-1 or EMCV resulted in complete lethality in IRF-7-/- mice compared to no change in IRF-3-/- mice compared to control. Examination of serum revealed a deficit in IFNα production in IRF-7-/- mice; no deficit was demonstrated in IRF-3-/- mice following intravenous HSV-1 challenge.

These results led to a revised model that defined IRF-7 as the "master regulator" of type I IFN and minimized the impact of IRF-3 (69). The new model proposes that IRF-7, expressed at low basal levels, is activated by viral infection and forms either a homodimer or heterodimer with IRF-3 driving production of type I IFN (173-174). In the absence of IRF-3, IRF-7 expression is sustained by weak activation of ISGF3 through spontaneously produced type I IFN (202). Low level IRF-7 expression partially

compensates for the loss of IRF-3, producing sufficient type I IFN to initiate the interferon response. Later, infiltration of hematopoietic immune cells expressing IRF-7 constitutively produces sufficient type I IFN to control the infection *in vivo* (69, 106). In contrast, the loss of IRF-7 results in initial IFNβ production via IRF-3, but no subsequent amplification of the type I IFN response (69). The resulting deficit in type I IFN production permits increased lethality in IRF-7 mice following intravenous viral challenge. Together, the data suggest IRF-7 plays a critical role in control of HSV-1 infection; in contrast, IRF-3 has only a minimal impact during HSV-1 infection both *in vitro* and *in vivo*.

However, data from several studies indicate the impacts of IRF-3 and IRF-7 on HSV-1 replication may not be so clear. In the absence of IRF-3 or IRF-7, HSV-1 replication is unaffected compared to wild-type (WT) cells (69). One possible explanation is that IRF-3 is specifically targeted by the virus to prevent induction of the immune response. For example, in the absence of viral gene expression, UV-inactivated HSV-1 induces IRF-3 activation and IFN induction to a higher levels than live virus, implying manipulation of IRF-3 mediated recognition (31, 158). HSV-1 ICP0, a multifunctional viral antagonist, has been shown to also interfere with IRF-3 activity via its N-terminal RING finger domain, an E3 ubiquitin ligase (15, 113). Studies demonstrated that HSV-1 prevents nuclear translocation of IRF-3 following co-infection with Sendai virus in an ICP0 dependent manner(126). Similarly, ICP0 has been shown to recruit IRF-3 and CBP/p300 to nuclear foci away from host chromatin, resulting in reduced IFNβ production (128). In addition, HSV-1 vhs and ICP27 have also been implicated in antagonizing the activity of IRF-3 (113, 125).

Goals of the Thesis

In recent years, there has been increased interest in the pathways that induce type I interferon in response to viral challenge. The discovery of RIG-I and the toll like receptors initiated examination of the pathogen recognition pathways and led to the discovery of new sensors, adaptors, and signaling molecules involved in type I IFN induction. IRF-3 is central to these newly discovered pathways; it provides a critical signaling component required for IFNβ induction and an efficient type I IFN response to viral infection. In the absence of IRF-3, the pathogen recognition pathways would be predicted to have delayed and deficient type I IFN production. The result would be increased susceptibility to viral infection.

The first goal of this thesis was to fully evaluate the impact of IRF-3 dependent pathways on HSV-1 replication *in vitro*. IRF-3 has been shown to play a critical role in the type I IFN cascade initiated by the early pathogen recognition pathways. Therefore, the loss of IRF-3 was predicted to impact the type I IFN response resulting in increased viral replication *in vitro*. However, the loss of IRF-3 had been previously shown to have no impact on viral replication in mouse embryonic fibroblast (MEFs), thus implying a minimal role for IRF-3 in the IFN response to HSV-1. Yet, HSV-1 entry has been shown to activate IRF-3 and the virus encodes viral proteins that antagonize IRF-3 activity; together, these results suggest that IRF-3 does impact HSV-1 infection. In order to address this apparent contradiction, immune cells lacking IRF-3 were challenged with HSV-1 *in vitro*. Immune cells were predicted to have a more vigorous immune response and the impact of IRF-3 on HSV-1 infection might be more discernable in these cell types.

The second goal of the thesis was to determine the role of IRF-3 on *in vivo* HSV-1 infection. IRF-3 had been previously shown to have no impact on survival following intravenous infection with HSV-1. However, intravenous challenge likely bypasses the physiologically relevant cell types for *alphaherpesvirus* infection: the epithelia and neurons. Therefore, examination of the IRF-3 deficient mice via the ocular route of infection permits evaluation of viral replication, trafficking of the virus, and the establishment of latency *in vivo*. Intracranial challenge with HSV-1 permits examination of lethality and viral fitness in the CNS.

The final goal of the thesis was to determine the pathways involved in recognition of HSV-1. While IRF-3 signaling has been implicated in sensing HSV-1 infection *in vitro*, the recognition pathway utilized by the host has not been identified. In order to determine the pathways involved in HSV-1 recognition, immune cells and mice lacking components of the RLR, TLR, and DNA sensing pathway were challenged with HSV-1. The loss of one or more of these components would be predicted to have a significant impact HSV-1 replication *in vitro* or *in vivo*.

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Chapter II

Control of herpes simplex virus replication is mediated through an IRF-3 dependent pathway.

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ABSTRACT

Type I Interferon (IFN) cascade is critical in controlling viral replication and pathogenesis. Recognition pathways triggered by viral infection rapidly induce the type I IFN cascade, often in an interferon regulatory factor 3 (IRF-3)-dependent fashion. This dependence predicts that loss of IRF-3 would render early recognition pathways inoperative and thereby impact virus replication, but this has not been observed previously with HSV-1 *in vitro*. In this study, HSV-1 infected IRF-$3^{-/-}$ bone marrow-derived dendritic cells (BMDCs) and macrophages (BMM) supported increased HSV-1 replication compared to control cells. In addition, IRF-3-deficient BMDCs exhibited delayed type I IFN synthesis compared to control cells. However, while IFN pretreatment of IRF-$3^{-/-}$ BMDCs resulted in reduced viral titers, a far greater reduction was seen following IFN treatment of wild-type cells. This suggests that even in the presence of exogenously supplied IFN, IRF-$3^{-/-}$ BMDCs are inherently defective in control of HSV-1 replication. Together, these results demonstrate a critical role for IRF-3 mediated pathways in controlling HSV-1 replication in cells of the murine immune system.

INTRODUCTION

Herpes simplex virus type I (HSV-1) is a ubiquitous human pathogen with high seroprevalence in adults (51). HSV-1 is associated with numerous human diseases ranging from the common cold sore in immune-competent individuals, to herpetic encephalitis in neonatal and immunocompromised hosts. A member of the Alphaherpesvirus family, HSV-1 exhibits two distinct phases of infection (49). Acute infection typically occurs at peripheral epithelial sites and is characterized by lytic infection and spread. In contrast, the virus shifts from lytic to latent infection in sensory neurons which is characterized by limited gene expression and the persistence of viral genomes in a transcriptionally active state. Following certain stimuli, periodic reactivation of latency occurs, and may result in shedding of infectious virus at the initial site of acute infection. Reactivation may also be associated with immunopathological diseases, most notably ocular herpetic stromal keratitis.

A role for interferons (IFNs) in controlling viral replication is well-established. In recent years, viral research has focused on cellular recognition of pathogen-associated molecular patterns (PAMPs) and subsequent IFN induction, leading to the discovery of toll-like receptors (TLRs) and retinoic acid inducible gene 1 (RIG-I) -like sensing molecules (18). Such molecules respond to several virally-derived PAMPs. These include MDA-5 and TLR-3 which recognize double-stranded RNA (13, 24), DAI and TLR-9 which recognize double-stranded DNA (14, 45), TLR-7 which recognizes single-stranded RNA (9), and RIG-I which recognizes triphosphate and double-stranded RNA (16, 31, 53). Subsequent work identified the adaptor molecules necessary for antiviral pathway signaling, including MyD88, TRIF and IPS-1 (19, 46, 52). Not surprisingly,

numerous gene products from viruses such as HCV, WNV, influenza and vaccinia have been identified to antagonize these pathways and serve to promote viral replication and virulence by degradation, interference, or sequestration of early recognition components (3, 11).

These newly-identified recognition pathways utilize IRF-3, IRF-7 and NFκB to induce IFN transcription through cognate binding sites on the IFN-β promoter (38). During initial induction of IFN, IRF-3 and NFκB, which are constitutively expressed, become activated and translocate to the nucleus where they bind the IFN-β promoter to form the IFN enhancesome (54). The initial IFN-β produced acts upon the IFNαβ receptor (IFNAR) in both an autocrine and paracrine manner to up-regulate interferon stimulated genes (ISGs), most notably IRF-7 (38). In concert with IRF-3, IRF-7 amplifies and facilitates expression of the full type I IFN cascade. In the absence of IRF-3, IFN-β production is reduced but IFN-α levels remain normal, suggesting that IRF-7 activity can compensate for the loss of IRF-3 (15). In contrast, IRF-7 deficiency results in significant reduction in serum IFN levels with a corresponding increase in susceptibility to virus infection. IRF-7 was therefore dubbed "the master regulator" of type I IFN-dependent immune responses (15). IRF-$7^{-/-}$ mice challenged with HSV-1 showed increased mortality compared to control and IRF-$3^{-/-}$ mice, but no increases in viral titers were observed in IRF-3- or IRF-7-deficient cells *in vitro* (15). A possible explanation for this lack of phenotype *in vitro* is that HSV-1 may control IRF-3 activation so thoroughly that this pathway is neutralized during infection. UV-inactivated HSV-1 induces IRF-3 dimerization and activation, leading to IFN induction, suggesting that very early events in infection are responsible for triggering this cascade in the absence of viral

gene expression (6, 23). ICP0, an immediate early gene of HSV-1, interacts with IRF-3 and plays a critical role in preventing the induction of the IFN response (10, 23, 27, 28, 30, 42). Additional HSV genes such as the virion host shut-off protein, ICP34.5, and ICP27 also interfere with the activity of IRF-3 (23, 26, 48). However, the increased susceptibility of IFN receptor knockout mice to HSV-1 compared to wild type mice suggests that despite so many genes regulating this pathway, the virus does not maintain total control over the type I IFN cascade (22, 32). In addition, numerous recognition molecules have been implicated in HSV-1 identification and the subsequent immune response (for example TLR-3, TLR-2, TLR-9, RIG-I), but the loss of any of these components does not result in any significant increase in viral replication *in vitro* (21, 35, 37, 55).

In these studies, we examined the impact of IRF-3 mediated pathways on HSV-1 replication using cells from IRF3-deficient (IRF-$3^{-/-}$) mice. The absence of IRF-3 was predicted to preclude the function of early recognition pathways and thereby impact HSV-1 replication. No changes in HSV-1 replication in IRF-3-deficient mouse embryonic fibroblasts (MEFs) had been observed previously, but we reasoned that relative to MEFs, cells of the immune system might induce more vigorous IRF-3-dependent antiviral responses, manifesting with a significant impact upon viral replication. Using IRF-3 deficient bone marrow-derived dendritic cells (BMDCs) and macrophages (BMM) we have demonstrated that IRF-3 mediated pathways are critical for control of HSV-1 replication. Moreover, control of HSV-1 replication is dependent on the type I IFN cascade in these cell types induced via IRF-3 mediated pathways.

METHODS

Cells and viruses. Viral stocks were grown and titers were determined on Vero cells (34). HSV-1 wild-type strain KOS was the background strain for this study (41).

BMDCs were generated from 6-8 week old C57BL/6 (Charles River Laboratories, Willmington, MA) or 129S6 (Taconic, Germantown, NY) mice (25, 56). Briefly, bone marrow was flushed from femurs of mice and cells were cultured as described below. For generation of BMDCs, bone marrow was cultured in RPMI with 10% fetal calf serum, Glutamax, Na pyruvate, non-essential amino acids, 250 U/ml penicillin, 250 U/ml streptomycin, and 2% GM-CSF for 6-8 days at 37°C. BMDCs were then collected, counted, and aliquoted for infection at several MOIs by the addition of virus in a minimal volume of medium for 30 minutes at 37°C. Cells were then spun at low speed, inocula removed, washed, resuspended, and plated in 35-mm wells for the duration of the experiment. BMDCs were also generated from mice deficient in IRF-$3^{-/-}$ (15), IRF-$7^{-/-}$ (15), STAT-$1^{-/-}$ (29) (Taconic, Germantown, NY), IFN-αβγ $R^{-/-}$ (AG129) (47).

Bone marrow macrophages were cultured as described (56). Briefly, bone marrow was cultured in DMEM supplemented with 10% fetal calf serum, 5% heat-inactivated horse serum, 20% L-929 conditioned medium, 250U/ml penicillin, 250U/ml streptomycin for 7 days on non-tissue culture treated plates. At day 7, cells were washed with a 0.02% EDTA solution, collected, and counted. The cells were plated in 35-mm wells and rested for three days. The BMM were infected at MOIs of 0.01 and 1 by the addition of virus in a minimal volume of medium for 30 minutes at 37°C, removal of inoculum, and followed by the addition of complete medium.

IFN-β ELISA. Bone marrow-derived dendritic cells were mock-treated or infected at an MOI of 5 with HSV-1 and cultured in 1 ml of medium. Cultured supernatants were harvested at 3, 6, 9, and 12 hours post infection and spun at low speeds to remove cells. Supernatants were stored at -20°C before assay of IFN-β in the medium using 50 μl of harvested medium in a mouse IFN-β enzyme-linked immunosorbent assay (ELISA) as described in the kit protocol (PBL Biomedical Laboratories, Piscataway, NJ).

Antibody blockade. MAR1-5A3, an IgG_1 monoclonal antibody specific to the IFN-α receptor (Leinco Technologies, St. Louis, MO) was utilized as described (39). Briefly, after infection, cells were plated in 1 ml of complete medium with 5μg/ml of MAR1-5A3 for the duration of the experiment. At specified times, cells were harvested and titered on Vero cells under methylcellulose.

Mixing Experiment. BMDCs were collected, counted, and aliquoted for infection. WT and IRF-$3^{-/-}$ BMDCs were mixed at a ratio of 1:1 such that cell numbers equaled those of non-mixed controls. The mixed and non-mixed populations were then immediately infected as previously described.

IFN-β pretreatment. BMDCs were treated for 16 hours with 100 U/ml mouse IFN-β (PBL Biomedical Laboratories, Piscataway, NJ) or mock in PBS. BMDCs were then collected, counted, and aliquoted for infection as previously described. No additional IFN-β was added after infection.

Statistics. All statistical calculations were determined by Student's *t* test and are relative to control cells unless otherwise stated.

RESULTS

Control of HSV-1 replication in BMDCs is IRF-3-dependent *in vitro*. A previous study using IRF-3- and IRF-7-deficient MEFs demonstrated that the absence of either signaling molecule did not significantly alter HSV-1 replication (15). Work performed in this laboratory is in agreement with these previous observations (Fig. 2.1). Additional experiments with IRF-3/IRF-7 double deficient MEFs also demonstrated no change in HSV-1 replication (Fig. 2.2). BMDCs were chosen for infection in this study due to their function as immune sentinels, their strong responses to IFN, and their critical role in controlling HSV-1 infection *in vivo* (17, 43, 44). IRF-$3^{-/-}$ BMDCs yielded at least 10 times more HSV-1 replication than control cells at both 24 and 48 hours post infection at each MOI tested (Fig. 2.3). In contrast, HSV-1-infected IRF-$7^{-/-}$ BMDCs did not yield any increased viral titers compared to wild-type control BMDCs. These results suggested that pathways for control of HSV-1 replication in BMDCs are dependent on IRF-3, but independent of IRF-7.

BMM require IRF-3 for control of HSV-1 replication. Primary bone marrow macrophages were infected in order to further assess the role of IRF-3 mediated pathways in immune cells (2, 5). These adherent BMMs were also tested to exclude the possibility that the replication pattern of HSV differed between MEFs and BMDCs because of their adherence and non-adherence to plastic substrates in culture. The results, however, demonstrated that the pattern of viral replication in the IRF-$3^{-/-}$ BMMs resembled that seen in BMDCs, with increased viral yields as compared to control cells (Fig. 2.4). 10- to 100-fold increases in viral yields were demonstrated at both 24 and 48 hours post

infection at MOIs of 0.01, 1, and 5 (data not shown). Interestingly, IRF-$7^{-/-}$ BMM also supported increased viral replication. In contrast to BMDCs, IRF-7 deficient BMMs permitted a 10- to 100-fold increase in viral replication compared to controls at 24 and 48 hours post infection. This increase in viral titers was greater in magnitude at the lower MOI, but the impact of IRF-7 loss on HSV replication in BMMs was less than the impact of loss of IRF-3. These data suggest a role for both IRF-3 and IRF-7 in control of HSV-1 replication in BMM. BMDCs, however, have no requirement for IRF-7 in controlling HSV-1 replication, demonstrating difference in the innate immune response between macrophages and dendritic cells. Overall, in both cell types, IRF-3 mediated pathways are required to control HSV-1 replication *in vitro*.

BMDCs lacking interferon receptors permit increased viral replication in a STAT-1 dependent manner. Having identified a role for IRF-3 mediated pathways in controlling HSV-1 replication in BMDCs and BMMs, focus was shifted to differentiating between 2 non-mutually exclusive mechanisms by which IRF-3 could be controlling HSV-1 replication. First, it is possible that IRF-$3^{-/-}$ BMDCs have delayed or reduced type I IFN responses, disrupting the type I IFN cascade, and resulting in increased viral replication. Second, it is possible that other IRF-3-dependent processes or gene products are directly controlling HSV-1 replication. To address these possibilities, BMDCs lacking both Type I and Type II IFN (αβ and γ receptors) were infected with HSV-1 (Fig. 2.5). These cells lack IFN binding and signaling, but contain IRF-3, and thereby maintain elements of the early recognition pathway via IRF-3 dependent gene expression. The IFN receptor-deficient BMDCs permitted increased viral growth in a similar fashion to IRF-$3^{-/-}$

BMDCs and suggested that the type I IFN cascade was responsible for controlling HSV-1 replication (Fig. 2.4). Similar increases in viral replication were also seen in STAT-1$^{-/-}$ BMDCs. Together, these data confirm that viral replication is significantly limited in these cell types through IFN-driven STAT-1 signaling. While these data do not completely exclude other IRF-3 dependent processes, the results strongly suggest that the increased viral yields in IRF-3$^{-/-}$ BMDCs and BMMs are due to a delayed or defective type I IFN cascade.

IRF-3 deficient BMDCs have a defect in IFN-β induction compared to WT control cells. IFN-β plays a critical role in inducing an antiviral state and controlling viral infection (18). A deficit or a delay in IFN-β induction would likely allow increased viral replication, as seen in IRF-3$^{-/-}$ BMDCs. In order to examine this question, IFN-β protein levels were determined by ELISA in control and IRF-3$^{-/-}$ BMDCs following infection with HSV-1 (Fig. 2.6). BMDCs were infected at an MOI of 5 to ensure uniform infection and minimize the contribution of bystander IFN. Even at this high MOI, IRF-3$^{-/-}$ BMDCs yielded a statistically significant increase in HSV-1 titer at 12 and 24 hours post infection. Examining IFN-β protein, IRF-3$^{-/-}$ BMDCs exhibited decreased and delayed IFN-β production relative to wild-type control BMDCs. WT BMDCs produced detectable levels of IFN-β as early as six hours post-infection and continued to escalate at nine and twelve hours post infection. In contrast, IRF-3 deficient BMDCs only produced measurable levels at twelve hours post-infection, suggesting a defect in the initiation of IFN-β production. The IRF-3$^{-/-}$ BMDCs were, however, capable of producing IFN-β late

in the experimental infection, thereby potentially allowing control of viral replication at these later times.

IFNαR-blocking antibody augments viral growth in wild-type control and IRF-3-deficient BMDCs. To demonstrate that production of type I IFN was a primary defect, WT and IRF-3$^{-/-}$ BMDCs were infected and then treated with antibodies that block the IFN-α receptor (IFNαR) or control IgG1 antibody (Fig. 2.7 and data not shown). We postulated that if the restriction of HSV-1 replication in this system was dependent on type I IFN induction then WT and IRF-3 deficient BMDCs should yield similar viral titers in the presence of the blocking antibody. Control IgG1 had no impact on viral replication in either cell type (data not shown). In contrast, the addition of IFNαR blocking antibodies allowed both wild-type and IRF-3$^{-/-}$ BMDCs to produce higher yields of HSV-1 such that viral growth curves for these two disparate cell types were similar under these conditions (Fig. 2.7). It was also notable that untreated IRF-3$^{-/-}$ BMDC cultures yielded similar titers as antibody-treated BMDCs at 24 hours post infection. In contrast, by 48 hours post infection, antibody-treated IRF-3$^{-/-}$ BMDCs yielded 10-fold more virus than untreated cultures. Together, these data demonstrate that the type I IFN cascade is responsible for controlling HSV-1 replication in wild-type BMDCs, and that at late time points, IRF-3-deficient BMDCs can exert partial type I IFN- dependent control of HSV-1 replication.

IFN induction from wild-type BMDCs fails to restore control of HSV-1 replication to IRF-3 deficient BMDCs *in vitro*. The preceding data suggested that IFN induction

was defective in IRF-3 deficient BMDCs, but that these cells were still capable of controlling viral infection once the type I IFN cascade had been initiated. The question arose therefore, if the initial IFN induction and synthesis were restored, could IRF-$3^{-/-}$ BMDCs limit HSV-1 replication to levels seen in wild-type control cells? We therefore investigated whether bystander IFN, produced by WT cells, could restore control of viral replication to IRF-$3^{-/-}$ BMDCs by mixing them in culture at a 1:1 ratio. The mixed cell population was then infected with HSV-1 at an MOI of 0.01 and viral replication measured (Fig. 2.8). Viral growth kinetics under these conditions were intermediate between those observed in wild-type (low viral growth) and IRF-$3^{-/-}$ (high viral growth) BMDCs. At 48 hours post infection, the mixed BMDC population gave a 10-fold increase in viral yield over wild-type cells alone, and a 10-fold decrease in viral yield over IRF-$3^{-/-}$ BMDCs alone. The results show that IRF-$3^{-/-}$ BMDCs are incapable of controlling viral replication even in the presence of bystander IFN induced by viral infection of WT cells. Another possibility, although less likely, is that the presence of IRF-$3^{-/-}$ BMDCs resulted in a reduced total type I IFN concentration thereby permitting increased replication in WT BMDCs. In either case, HSV-1 replication of IRF-3 deficient BMDCs was not limited in the context of bystander cell-produced IFN.

IRF-3 deficient BMDCs primed with IFN partially restore control of HSV-1 replication. The results from the cell mixing experiments suggested that IRF-$3^{-/-}$ BMDCs were unable to respond fully to IFN production by WT cells. However, the ability to generate a delayed IFN-β response coupled with the IFN-dependent decrease in viral titers at late time points suggested that IRF-$3^{-/-}$ BMDCs were capable of inducing the type

I IFN cascade, but with low efficiency. One possible model is that cells lacking IRF-3 are inherently slowed in their response to IFN, and need additional time to properly prime in order to fully control HSV-1 replication *in vitro*. To test this, WT and IRF-3$^{-/-}$ BMDCs were pretreated overnight with IFN-β, challenged with HSV-1, and viral yields measured (Fig. 2.9). IFN pre-treatment of IRF-3$^{-/-}$ BMDCs significantly decreased HSV-1 replication as compared to untreated IRF-3$^{-/-}$ cells with a greater than 100-fold decrease in viral titers at 48 hours post infection. Titers observed were comparable to those in untreated WT control BMDCs. However, pretreatment of WT control cells resulted in further decreases in viral replication, to levels at, or below, the level of detection. These results together suggest that IRF-3 deficient BMDCs were capable of strongly responding to IFN, but the overall immune response in controlling HSV-1 replication was still defective compared to WT control cells.

DISCUSSION

Despite mice or cells lacking IFN receptors being significantly more susceptible to viral infection (22, 32), loss of IRF-3 and IRF-7 had surprisingly little impact on HSV-1 replication *in vitro* (15). Several groups have suggested the lack of a growth phenotype in IRF3$^{-/-}$ cells may be due to HSV-1 maintaining strict control over IRF-3-dependent pathways through various viral genes including ICP0, ICP27, ICP34.5 and *vhs*, thereby neutralizing the impact of IRF-3 mediated pathways (6, 10, 23, 26-28, 30, 35, 42). In this study, we have demonstrated that HSV-1 replication was controlled in an IRF-3-dependent manner in two types of immune cells. This control was dependent on type I IFN and STAT-1 signaling with a primary defect in IFN production in IRF-3$^{-/-}$ cells. Even in the presence of exogenously-supplied IFN, however, HSV-1 replication was only partially controlled in IRF-3$^{-/-}$ BMDCs. Overall, the data presented provide evidence that IRF-3 mediated pathways have a significant impact on HSV-1 replication in certain cell types.

Previous studies examining HSV-1 and IRF-3$^{-/-}$ used highly permissive MEFs, whereas in this study dendritic cells and macrophages were chosen. Given the roles of dendritic cells and macrophages as sentinels of the immune system capable of controlling viral infection *in vivo,* it is likely that these cells induced a more vigorous immune response and were thereby less permissive to infection than MEFs (2, 5, 17, 43, 44). In the case of HSV, a virus with multiple mechanisms to subvert IFN responses, loss of IRF-3 can only manifest with increased viral titers in cells that respond strongly to IFN. This idea is supported by studies with West Nile virus (WNV) (12). Only at late time points, IRF-3$^{-/-}$ MEFs support nearly a 4 log increase in WNV titers compared to control

cells suggesting robust IRF-3 dependent responses in control MEFs late in infection. In contrast, examination of WNV in bone marrow macrophage cultures demonstrated increased viral replication in IRF-3$^{-/-}$ BMM immediately, as early as 24 hours post infection, and continued through 72 hours post infection (7). A similar difference in viral replication between MEFs and immune cells was reported with mouse norovirus (MNV) in the context of STAT-1 deficiency (50). These data support the hypothesis that immune cells have a more vigorous antiviral response than MEFs and loss of IRF-3 on viral replication may be more accurately measured in more restrictive immune cell types. This hypothesis is especially relevant to HSV-1, which relative to WNV and MNV, has more genes for IFN regulation, produces less dsRNA, and exhibits less sensitivity to type I IFN.

Not surprisingly, in the absence of IRF-3, BMDCs and BMM were unable to efficiently control HSV-1 replication (Figs. 2.3 and 2.4). While the IFN receptors are intact in these cells, the early recognition signaling likely cannot proceed efficiently without IRF-3, leading to a delay in the type I IFN cascade. Later, once secondary rounds of infection have begun, alternate recognition pathways, most likely mediated through IRF-7, can lead to the induction of type I IFN. This recognition by a secondary pathway is supported by the observed late production of IFN-β (Fig. 2.6) and the concomitant decreased viral replication in IRF-3$^{-/-}$ BMDCs at late time points (Fig. 2.7). While IRF-3 is constitutively expressed in both BMDCs and BMM, basal expression of IRF-7 varies according to cell type (33). Plasmacytoid dendritic cells (pDCs) constitutively express IRF-7 while IRF-7 expression is reduced in conventional BMDCs as compared to IRF-3. BMMs exhibited basal expression of both IRF-3 and IRF-7,

potentially explaining increased replication in IRF-7$^{-/-}$ BMM but not in IRF-7$^{-/-}$ BMDCs (8). Those studies observed a parallel trend in WNV replication in IRF-7$^{-/-}$ BMM and BMDCs as seen here with HSV-1. Yet, in both cell types, the presence of IRF-7 cannot compensate for the loss of IRF-3 mediated pathways. These results suggest that IRF-3 mediated pathways provide the major pathway of control of HSV-1 replication.

Together, our results demonstrate that the early recognition response through IRF-3- mediated pathways controls HSV-1 replication in BMDCs and lead to the following model (Fig. 2.10). Upon virus entry, an as-yet undetermined sensor recognizes HSV-1 and triggers a signaling cascade that activates IRF-3. IRF-3 activation leads to production of IRF-3 dependent gene products, type I IFN, and an ensuing type I IFN cascade, resulting in control of HSV-1 infection. In the absence of the type I IFN cascade, achieved by knockout (Fig. 2.5) or receptor blockade (Fig. 2.7), BMDCs are unable to control viral replication. Similarly, ablating IRF-3 and the early recognition response results in increased viral replication due to delayed and reduced IFNβ production (Fig 2.3, 2.4, 2.6). Exogenous IFN provided by bystander cells (Fig. 2.8) or pretreatment (Fig. 2.9) partially restores control of HSV-1 replication in IRF-3-/- deficient BMDCs, yet these cells remain defective in their control of HSV-1 replication compared to treated WT cells.

Several non-mutually exclusive possibilities exist to explain this persistent defect in the ability of IRF-3$^{-/-}$ BMDCs to control HSV-1 replication (Fig. 2.10, white squares). One possibility is a defective autocrine and paracrine IFN amplification response. While wild-type BMDCs quickly respond to IFN through STAT-1 and IRF-3 signaling pathways, IRF-3$^{-/-}$ BMDCs can only respond through STAT-1-dependent, IRF-3-

independent pathways. The absence of IRF-3 thereby severely decreases or ablates the expression of several gene products, including IFN-β, IFNα4 and IFNα5 (1), resulting in less robust IFN signaling. A second possibility is that IRF-3 dependent ISGs synergize with Type I IFN receptor dependent ISGs and control HSV-1 replication, but fail to be produced robustly in IRF-$3^{-/-}$ BMDCs. A third possibility is that virus recognition may be required to augment the ongoing immune response. IFN primed IRF-$3^{-/-}$ BMDCs may produce IFN effectors, but a lack of viral recognition signaling results in a delayed effector response from ISGs. IRF-$3^{-/-}$ BMDCs may therefore require HSV-1 recognition signaling through a secondary pathway before fully committing to a complete IFN effector response, and this delay could result in the observed increased viral replication, compared to wild-type controls.

Together, these data demonstrate that immune cells lacking IRF-3 are inherently defective in the control of HSV infection. These data, however, conflict with previously published *in vivo* data following intravenous (IV) infection (15). A possible explanation is that following IV infection, IFN was being produced by plasmacytoid dendritic cells (pDCs). pDCs, typically found in the lymph nodes away from the site of infection, are a major producer of type I IFN, and they rely on TLR-9 and IRF-7 pathways to induce IFN in response to HSV-1(4, 20, 40). Following IV infection, therefore, pDC production of type I IFN likely overcomes the IFN deficit and thereby is able to control HSV-1 replication in the absence of IRF-3. Previous *in vitro* studies in MEFs suggested a role for HSV-1 gene components in interfering with and neutralizing the activity of IRF-3 (10, 23, 26-28, 30, 36, 42, 48). In the cell types used in this study, heightened immune responses likely reduced the efficacy of one or more viral immuno-regulatory

components, or presented too great of a challenge for the viral activities to counter. Therefore, the efficacy of HSV-1 genes in antagonism of IRF-3 likely depends on the overall capacity of the infected cell to mount an immune response to the incoming virus. Ongoing experiments in our laboratory seek to determine the precise molecules responsible for HSV-1 recognition. As mentioned previously, several candidates in the early recognition pathways have been implicated (21, 35, 37, 55) and cells lacking these components are currently being tested for their ability to control viral replication in BMDCs. Furthermore, *in vivo* studies in IRF-3 deficient animals are currently underway in order to examine HSV-1 replication and pathogenesis in peripheral and neuronal tissues.

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Figure 2.1. *In vitro* replication in mouse embryonic fibroblasts. Primary MEFs were infected with wild-type HSV-1 at an MOI of 1 or 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are mean titers of three independent experiments.

Figure 2.2. *In vitro* replication in double deficient mouse embryonic fibroblasts. Primary MEFs lacking IRF-3 and IRF-7 were infected with wild-type HSV-1 at an MOI of 5, 1, or 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are mean titers of three independent experiments.

Figure 2.3. *In vitro* replication in bone marrow derived dendritic cells. Primary BMDCs were infected with wild-type HSV-1 at an MOI of 1 or 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are the mean titers of three independent experiments. *p value <0.05 **p value <0.01.

Figure 2.4. *In vitro* replication in bone marrow derived macrophages. Primary BMM were infected with wild-type HSV-1 at an MOI of 1 or 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are mean titers of four independent experiments. *p value <0.05 **p value <0.01***p value<0.001.

Figure 2.5. *In vitro* replication in BMDCs lacking IFN signaling. Primary BMDCs derived from wild-type, IFNαβγ Receptor deficient (AG129) or STAT-1 deficient mice were infected with wild-type HSV-1 at an MOI of 1 or 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are the mean titers of three independent experiments. *p value <0.05 **p value <0.01***p value<0.001.

Figure 2.6. IFN-β secretion by infected BMDCs. Primary BMDCs were infected with wild-type HSV-1 at an MOI of 5. At indicated times post infection, cells and supernatants were harvested. Cells were removed by low speed centrifugation and supernatants were assayed for IFN-β by ELISA. Results are shown in pg/mL and are mean totals from three independent experiments. Cells and supernatants were also assayed for viral titers at 6, 12, and 24 hours post infection and viral titers were assayed on Vero cells. Results shown are the mean titers of three independent experiments. *p value <0.05

Figure 2.7. IFNαR blockade in BMDCs. Primary BMDCs were infected with wild-type HSV-1 at an MOI of 0.01. Following infection, BMDCs were plated in media containing 5μg/mL IFNαR blocking antibody (MAR1-5A3) for the duration of the experiment. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are the mean titers of three independent experiments. *p value <0.05 **p value <0.01.

Figure 2.8. *In vitro* replication following mixing BMDC populations. Primary WT and IRF-3$^{-/-}$ BMDCs were mixed at a 1:1 ratio and infected at an MOI of 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are the mean titers of three independent experiments. *p value <0.05.

Figure 2.9. *In vitro* replication following IFN-β pretreatment of BMDCs. Primary WT and IRF-3$^{-/-}$ BMDCs were pretreated with 100U/mL mouse IFN-β for 16 hours. Cells were then infected with WT HSV-1 at an MOI of 0.01. At indicated times post infection, cells and supernatants were harvested and viral titers assayed on Vero cells. Results shown are the mean titers of three independent experiments. *p value <0.05 **p value <0.01.

Figure 2.10. Model for continued defect in IRF-3 deficient BMDCs. Post attachment, HSV-1 infection is recognized through an unknown sensor mechanism that leads to activation of IRF-3. The early recognition pathway mediates production of type I IFN and IRF-3 dependent interferon stimulated genes leading to the control of HSV-1 replication via the type I IFN cascade. However, pretreatment with IFN does not restore HSV-1 replication in IRF-3$^{-/-}$ BMDCs to WT levels. The continued defect is potentially due to three, non-exclusive mechanisms outlined in white squares: defective IFN amplification, defective antiviral trigger signaling, and IRF-3 dependent gene synergy with the antiviral response. One or more of these mechanisms leads to continued defect in the control of HSV-1 replication in IRF-3$^{-/-}$ BMDCs as compared to WT BMDCs after IFN treatment.

Figure 2.1. *In vitro* replication in mouse embryonic fibroblasts.

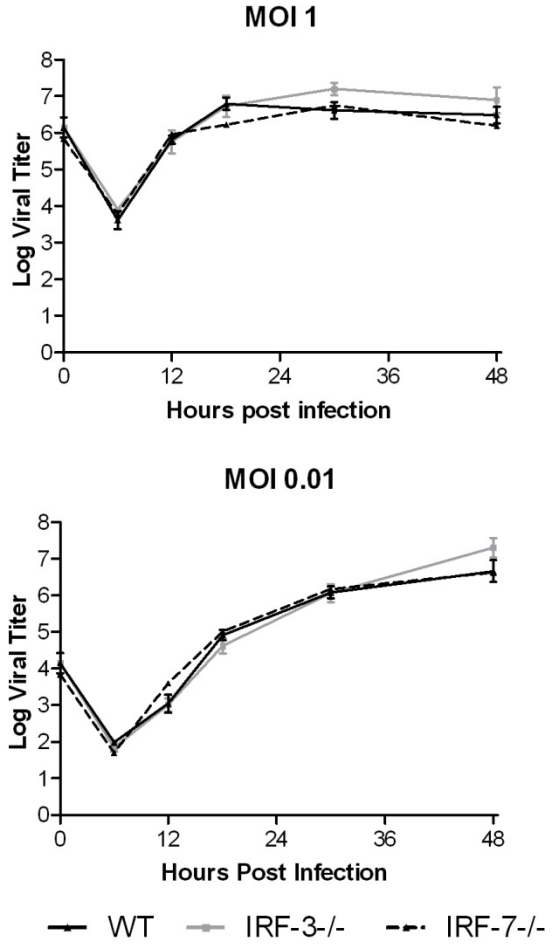


Figure 2.2. *In vitro* replication in double deficient mouse embryonic fibroblasts.

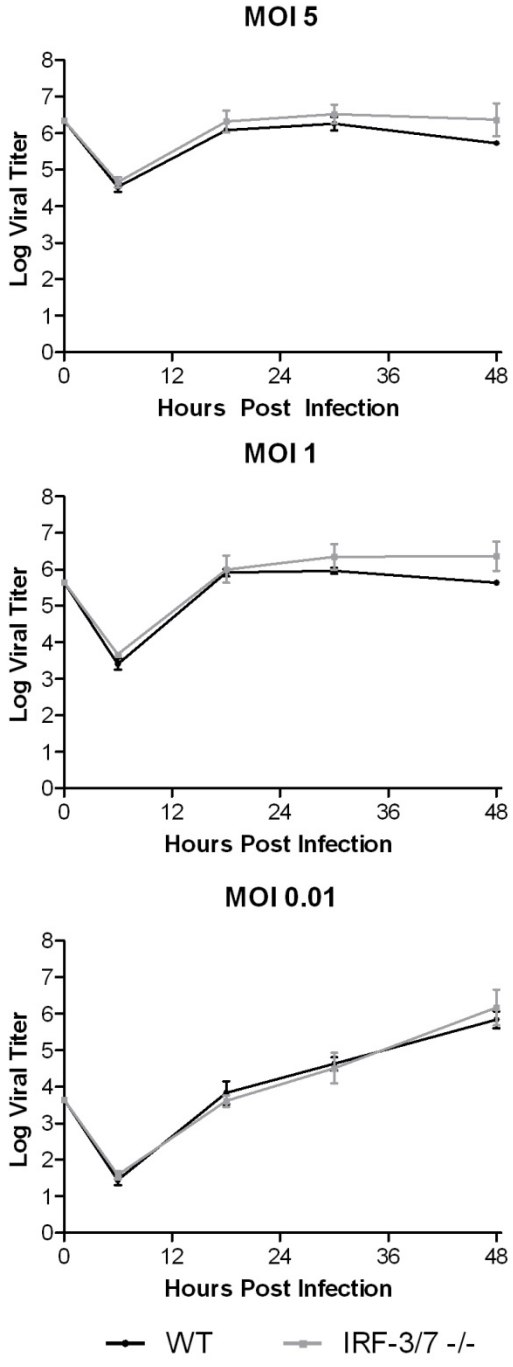


Figure 2.3. *In vitro* replication in bone marrow derived dendritic cells.

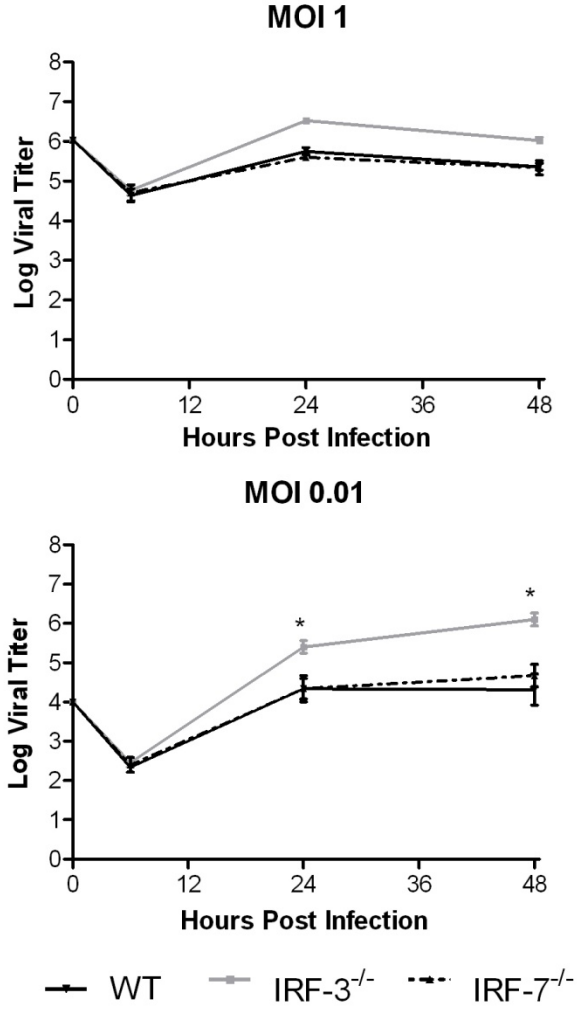


Figure 2.4. ***In vitro*** **replication in bone marrow derived macrophages.**

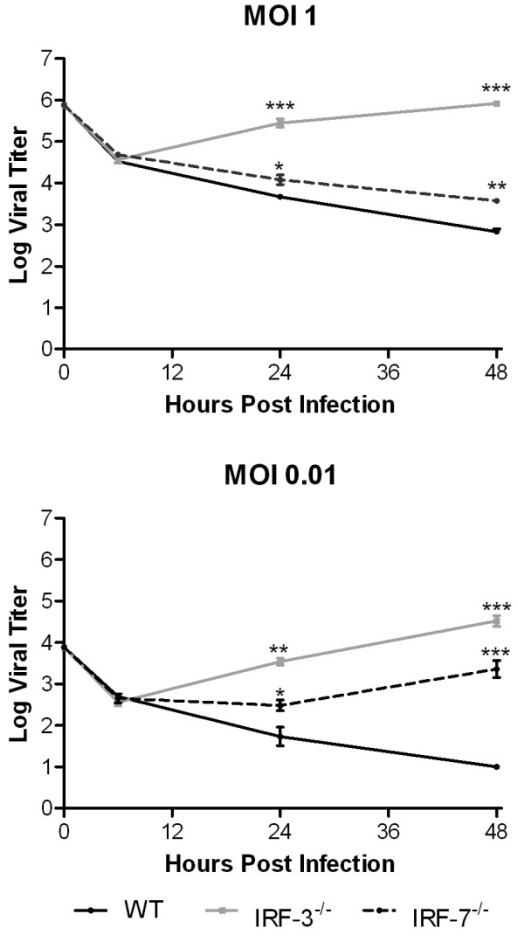


Figure 2.5. ***In vitro*** **replication in BMDCs lacking IFN signaling.**

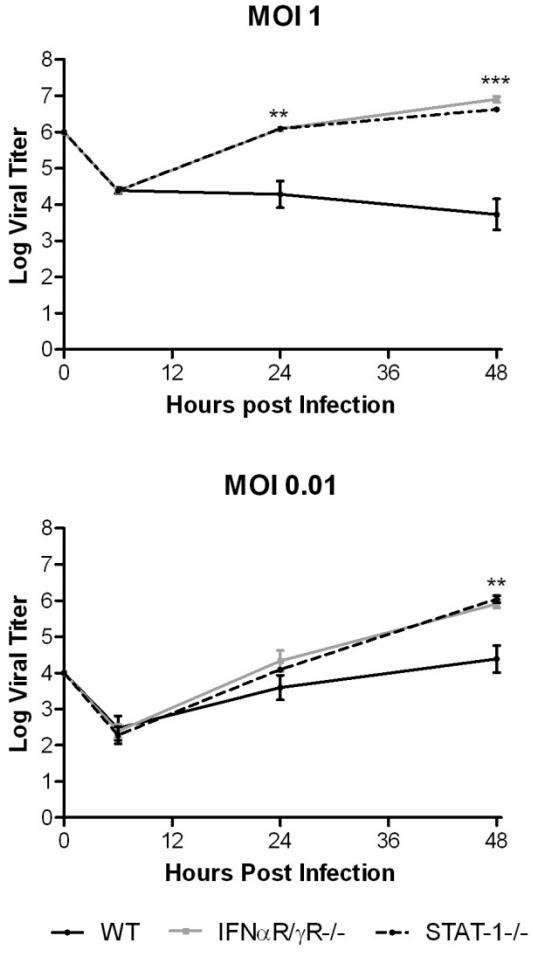


Figure 2.6. IFN-β secretion by infected BMDCs.

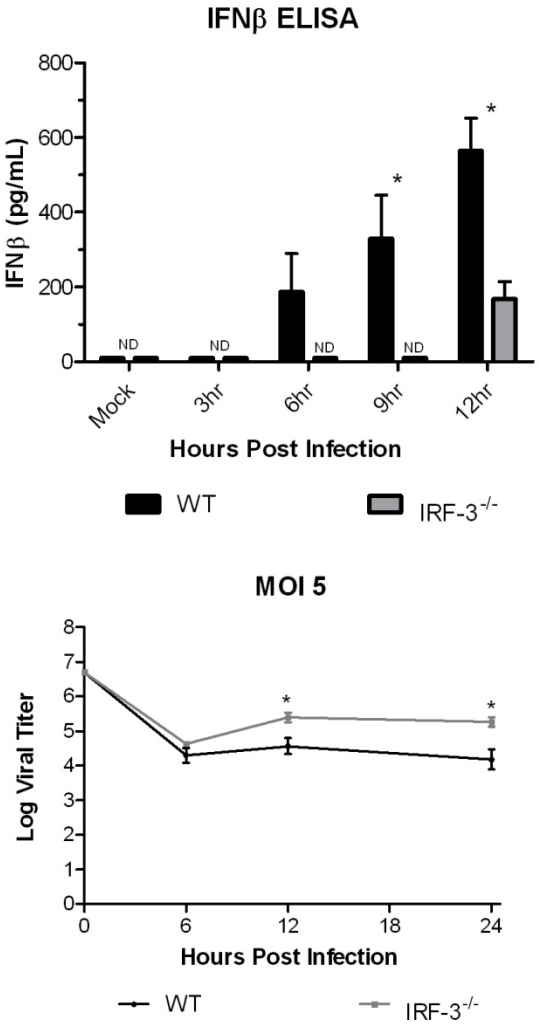


Figure 2.7. IFNαR blockade in BMDCs.

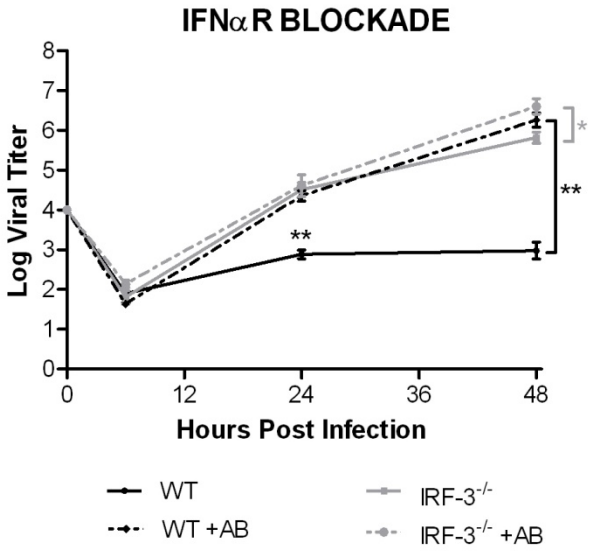


Figure 2.8. *In vitro* replication following mixing BMDC populations.

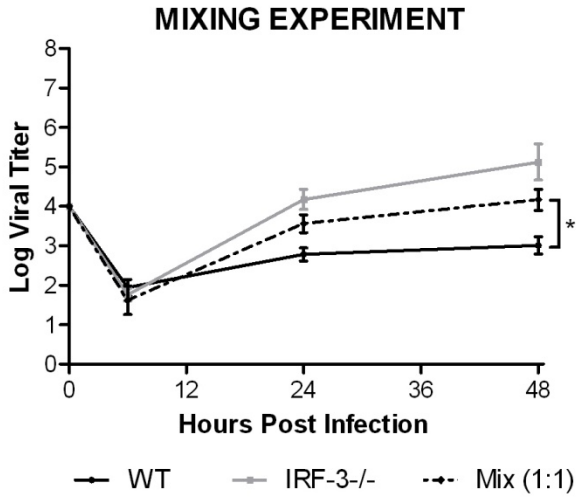


Figure 2.9. *In vitro* replication following IFN-β pretreatment of BMDCs.

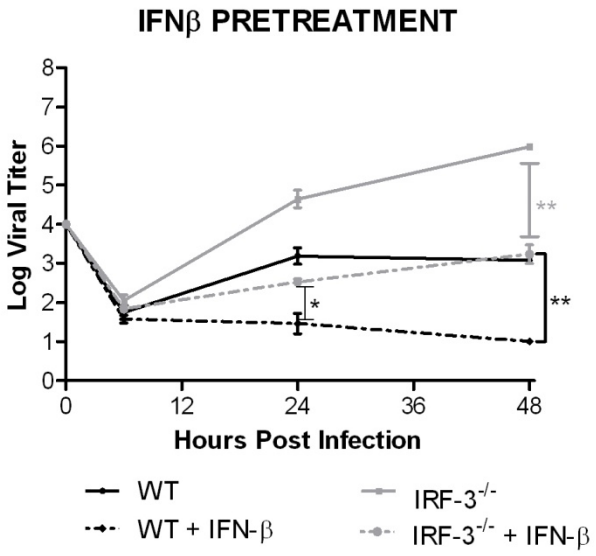
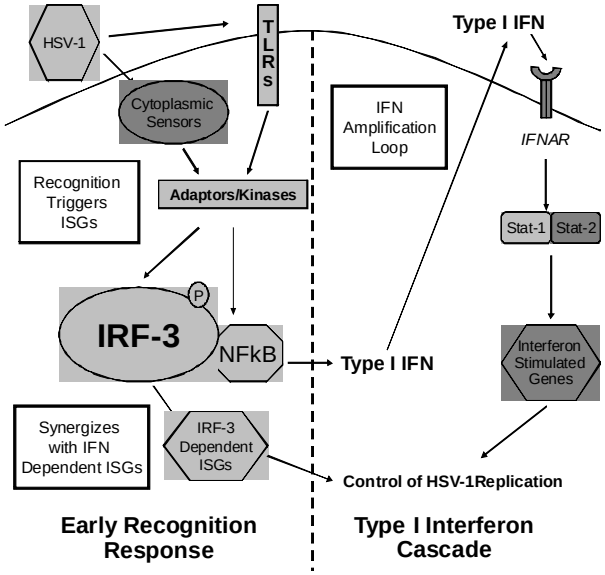


Figure 2.10. Model for continued defect in IRF-3 deficient BMDCs.



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Chapter III

IRF-3 mediated control of HSV-1 replication *in vivo*.

ABSTRACT

The initiation of the immune response at the cellular level relies on specific recognition molecules to rapidly signal viral infection via interferon (IFN) regulatory factor-3 (IRF-3) -dependent pathways. The absence of IRF-3 would be expected to render such pathways inoperative and thereby significantly impact viral infection. Unexpectedly, previous studies found no significant change in herpes simplex virus (HSV) pathogenesis in IRF-3$^{-/-}$ mice following intravenous HSV-1 challenge (Honda, K., et al., Nature 2005, 434:772-777). In contrast, the present study demonstrated that IRF-3$^{-/-}$ mice are significantly more susceptible to HSV infection via the corneal and intracranial routes. Increased viral replication and inflammatory cytokine production were observed in brain tissues of IRF-3$^{-/-}$ mice compared to control mice, with a concomitant deficit in production of both IFNβ and IFNα. These data demonstrate a critical role for IRF-3 in control of central nervous system infection following HSV-1 challenge. Furthermore, this work underscores the necessity to evaluate multiple routes of infection and animal models in order to fully determine the role of host resistance factors in pathogenesis.

INTRODUCTION

Herpes simplex virus type I (HSV-1) is a ubiquitous pathogen of the *alphaherpesvirus* family with high seroprevalence in the adult human population (64). Possessing two distinct phases, HSV-1 causes a life-long infection with an initial lytic stage followed by a shift to latency following trafficking to sensory neurons (63). Periodically, reactivation from latency occurs and is associated with numerous diseases ranging from the common cold-sore to ocular herpetic stromal keratitis (HSK), a leading cause of infectious blindness (25, 47). Reactivation events as well as primary infections are associated with herpes simplex encephalitis (HSE), a rare, but life threatening consequence of infection of the central nervous system (CNS) (62). Through recurrent infection in adults or maternal transmission to neonates, HSV-1 infects the brain and causes acute inflammation and significant pathological damage resulting in nearly 70% lethality if untreated (26, 62). In developed countries, HSV remains among the most common causes of viral encephalitis(60).

Studies in mouse models and clinical studies have underscored the importance of the immune response, especially type I IFN, in protection of the host from encephalitis (11, 15, 22, 68). In response to viral infection, type I IFN initiates a signaling cascade to stimulate the immune system and provide a first line defense against invading pathogens (50). Consisting of IFNβ and several forms of IFNα, type I IFN binds a receptor (IFNAR) to induce an anti-viral state through production of numerous interferon stimulated genes (ISGs) (19, 23, 53). In the absence of type I IFN signaling, mice are very susceptible to disseminated peripheral HSV-1 infection, leading to increased viral replication and increased mortality *in vivo* (28, 30, 45-46) .

In the CNS, type I IFN plays a critical role in control of viral infections . While peripheral tissues rely on plasmacytoid dendritic cells (pDCs) as the major IFN producing cells, the brain is largely devoid of this cell type (1-2, 54). Instead, the CNS relies on resident cells including neurons to produce and respond to type I IFN (10). In the absence of type I IFN receptors, mice are very susceptible to encephalitis caused by a variety of viral pathogens (9, 15, 22). Mice and humans with defects in type I IFN signaling were also found to be more susceptible to HSE than control groups (13). Together, these studies signal the importance of IFN signaling following CNS infection. Recent studies, however, have focused on the importance of type I IFN induction in limiting viral encephalitis. In particular, inborn disorders of IFN production, as well as TLR-3 mutations, render otherwise healthy individuals susceptible to HSE (5, 68). These data suggest that recognition pathways producing type I IFN in the CNS are as important as IFN signaling in controlling virally induced encephalitis.

Work on pathogen-associated molecular patterns (PAMPs) has revealed two major recognition pathways that lead to type I IFN production (3). The toll-like receptor (TLR) pathways sample the extracellular milieu via receptors on the cell surface and within endosomes (12, 18, 35). In contrast, the RIG-I like receptor (RLR) pathways utilize a variety of sensors to recognize nucleic acid PAMPs within the cytosol of infected cells (17, 58, 66). Each pathway utilizes a variety of adaptors and signaling molecules to induce type I IFN production (24, 59, 65), yet both pathways converge onto three common signaling molecules: IRF-3, IRF-7, and NFκB (52). Following activation via the upstream recognition pathways, these signaling components bind the IFNβ promoter

to form the "IFN enhancesome" (67). The IFNβ initially produced acts upon the IFNαβ receptor (IFNAR) in both an autocrine and paracrine manner. This leads to the induction of ISGs and the type I IFN cascade.

While NFκB is activated via independent adaptors, IRF-3 and IRF-7 were initially thought to be interchangeable (52). The formation of IRF-3/IRF-7 homodimers or heterodimers was necessary for binding specific regions of the IFNβ promoter and production of type I IFN (19-20). Examination of cells and animals deficient in IRF-3 or IRF-7, however, revealed distinct roles for the two signaling components. In the absence of IRF-3, mice challenged with HSV-1 showed reduced serum IFNβ production, but constant IFNα levels and the mice survived intravenous challenge (21). In contrast, IRF-7 deficiency resulted in reduced serum IFNα levels and a corresponding increase in mortality following HSV-1 intravenous infection. Therefore, IRF-7 was believed to compensate for the loss of IRF-3 and dubbed "the master regulator" of type I IFN dependent immune responses (21). Some recent studies, however, have indicated that the respective impacts of IRF-3 and IRF-7 on HSV-1 replication may not be so clear cut. For example, replication of HSV-1 in IRF-3- or IRF-7-deficient mouse fibroblasts was unaffected relative to wild-type cells (21). One potential explanation postulated in several studies is that IRF-3 is specifically targeted by the virus to prevent induction of the immune response. For example, in the absence of viral gene expression, UV-inactivated HSV-1 induces IRF-3 activation and IFN induction to a greater extent than live virus (7, 29, 44). Viral genes including ICP0, virion host shutoff protein, ICP34.5, and ICP27 have all been implicated in directly or indirectly targeting IRF-3 (14, 29, 37-

39, 42-43, 56, 61). Together, these data suggest that the virus targets IRF-3 and implies that IRF-3 can impact HSV-1 infection.

Recent studies from this laboratory demonstrated a significant increase in viral replication in immune cells in the absence of IRF-3 (40). The loss of IRF-3 resulted in increased viral replication in bone marrow-derived dendritic cells and macrophages due to delayed and deficient type I IFN production. In the current study, the role of IRF-3 *in vivo* was examined. Utilizing two routes of infection, via the cornea and through direct intracranial inoculation, several aspects of HSV-1 infection were evaluated, including viral replication, viral tropism, lethality, and cytokine production. The study confirmed previous results showing no significant impact of IRF-3 on replication in peripheral tissues (21). In contrast to previous studies, loss of IRF-3 had a significant impact on viral replication, lethality, and cytokine production in the brain following both cornea and intracranial routes of infection. Together, the results demonstrate that IRF-3 is a pivotal determinant of viral tropism and determines the outcome of HSV infection of the central nervous system.

MATERIALS AND METHODS

Cells, virus, and mice. Vero cells were used for production and determination of viral stock titers as previously described (49). The HSV-1 wild-type strains were strain 17 (HSV-1 17) and strain McKrae (HSV-1 McKrae) (36, 48). Mock-treated animals were inoculated with uninfected Vero cell lysates prepared in parallel to viral stocks. The mouse strains used were control C57B6 as WT mice and C57B6 IRF-3 deficient mice (IRF-$3^{-/-}$) (52) of either gender. Mice were housed in the Washington University School of Medicine barrier facility and infected in the Washington University School of Medicine biohazard facility. Mice were infected at between 6 and 8 weeks of age. Mice were euthanized, if necessary, in accordance with Federal and University policies.

Animal infection procedures. For corneal infection, mice were anesthetized intraperitoneally with ketamine (87mg/kg of body weight) and xylazine (13 mg/kg). Corneas were bilaterally scarified with a 25G syringe needle, and virus was inoculated by adding $2x10^6$ PFU HSV-1 in a volume of 5µl. Mice were sacrificed at specified times post infection for tissue harvest or observed daily for 21 days to evaluate survival.
For intracranial infections, mice were anesthetized as described above, and injected intracranially with 100 PFU, $1x10^5$ PFU of HSV 17 or mock in a volume of 20µl DMEM using a Hamilton syringe with a 26G needle. Mice were sacrificed at specific times post infection for tissue harvest or observed until day 21 post infection to evaluate survival.

Tissue titers. Following *in vivo* cornea infection, the following tissues were harvested and tittered as previously described (49): corneal swabs, periocular skin, trigeminal

ganglia, brain and brain stem. Briefly, tissues were harvested and stored at -80C until processing. Tissues were mechanically disrupted, sonicated and tittered via standard plaque assay on Vero cells.

Histological analysis. WT and IRF-3$^{-/-}$ mice were infected and harvested at day 3 and 5 post infection as described above. Briefly, mice were sacrificed and whole brains were harvested into 4 ml of 10% formalin solution for fixation. The brains were then sectioned sagittally and every tenth section stained using an anti-HSV-1 polyclonal antibody. Each section was divided into five regions (olfactory bulb, central brain, mid-brain cerebellum, and brain stem), and scored as either positive or negative for HSV antigen staining in a masked fashion. Total positive regions were then divided by total sections counted to obtain percent antigen positive regions.

Bead based cytokine analysis. Brains and brain stems were isolated and assayed following in infection *in vivo*. A single brain or brain stem was harvested from mice and mechanically disrupted in 1ml of phosphate buffered saline (PBS). Samples were then sonicated on ice twice for 30 seconds and centrifuged for 4 minutes at 1500rpm at 4°C. Supernatants were transferred to a 1.5mL eppendorf tube and centrifuged for 5 minutes at 7500rpm at 4°C. Supernatants were then transferred to new tube and diluted 1:1 with serum sample diluent (Bioplex Mouse Serum Sample Kit, Bio-Rad, Hercules, CA). The samples were then stored at -80°C until assayed. The Bio-Plex assay (Bio-Rad) was preformed as described in the kit protocol. Briefly, equivalent amounts of protein, as measured by Bradford assay, were added to each well of a multiplex mouse cytokine Bio-

Plex array. Cytokine concentrations were determined by comparison to a standard curve provided by Bio-Rad and the results are reported as pg/ml/μg protein. The results shown are the average from two experiments, with each experiment containing three or more mice per data point.

IFN ELISA. Following a high-dose intracranial infection, brains were harvested at 12, 18, and 48 hours post infection. The brains were mechanically disrupted and sonicated two times in 1ml PBS. Brain samples were then spun at 1.5K in a tabletop centrifuge. The supernatant were then harvested and spun at 7.5K in a mini-centrifuge for 10 minutes. The clarified supernatants were harvested and stored at -80°C until processing by ELISA. For both IFNβ and IFNα ELISA, 100μl of samples were assayed per kit protocol (PBL InterferonSource, Piscataway, NJ). Protein levels were normalized via Bradford assay and results were expressed pg IFN per mL per μg protein.

Real time RT-PCR of brain tissue. At the indicated time post-infection, brains were harvested into 2mL of Solution D (4 M guanidine thiocyanate, 25 mL of sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) (6) and stored at -80^{o}C. Total RNA was harvested as previously described (45) and resuspended in a small volume of nuclease-free water. cDNA was generated using the iScript cDNA synthesis kit as per kit protocol (Bio-Rad, Hercules, CA). PCR reactions were prepared with iQ SYBR green supermix (Bio-Rad), 5% acetamide, primers (IDT, Coralville, IA) and 2 □L cDNA. Each PCR was performed in duplicate, and each infection condition was replicated in at least 4 mice from 3 independent experiments. Actin primer sequences F-

5’:TGGTACGACCAGAGGCATACAG; R-5’:CCAACTGGGACGACATGGAG. IFN-□primer sequences: F-5’:CAGCTCCAAGAAAGGACGAAC; R-5’: GGCAGTGTAACTCTTCTGCAT.

Statistics. Statistical calculations were determined by Student’s *t* test and are relative to WT control group unless otherwise stated herein. Statistical analysis of survival curves utilized the log-rank test.

RESULTS

IRF-3$^{-/-}$ mice have no defect in controlling HSV-1 infection following peripheral cornea infection. Following infection with 2x10^{6} PFU HSV-1 strain 17, examination of corneal eye swabs from IRF-3$^{-/-}$ and WT mice demonstrated no significant increase in viral replication following infection (Figure 3.1A.) Similarly, periocular skin from IRF-3$^{-/-}$ mice had no major change in HSV-1 titers compared to control (Figure 3.1B); these data suggest that IRF-3 plays only a minor role in HSV-1 control in the epithelial cells of the cornea and periocular skin. Examination of the trigeminal ganglia also demonstrated no significant increase in viral replication IRF-3$^{-/-}$ mice (Figure 3.1C). Additionally, no change was observed in terms of lethality between IRF-3$^{-/-}$ and control; however, previous reports have found HSV-1 17 to be less virulent in the C57B6 strain of mice and no mice died in the experiments preformed here (33). Reactivation was also shown to be similar between WT and IRF-3$^{-/-}$ suggesting that IRF-3 deficiency had minimal impact one HSV-1 latency (Figure 3.2). Together, the data supported the previous findings by Honda et al. and suggest that IRF-3 mediated pathways play only a minor role in controlling HSV-1 during peripheral infection (21).

IRF-3$^{-/-}$ mice show increased mortality following cornea infection and an associated increase in viral replication in the brain stem. As mentioned previously, HSV-1 strain 17 has minimal lethality and neuroinvasiveness in C57B6 mice following peripheral challenge (33). To evaluate lethality and viral replication in the brain, a neurovirulent and neuroinvasiveness strain of virus, HSV-1 McKrae, was utilized to determine differences between WT and IRF-3$^{-/-}$ mice. As reported with HSV-1 strain 17, no change

in viral titers was observed following HSV-1 McKrae infection in the cornea, the periocular skin, or the trigeminal ganglia (Fig 3.3A-B, data not shown). However, infection with the neurovirulent virus resulted in approximately 50% survival in wild-type mice and less than 10% survival in IRF3$^{-/-}$ mice, demonstrating significantly ($p<0.05$) increased susceptibility of IRF3$^{-/-}$ mice (Fig. 3.4A). Evaluating viral titers, IRF-3$^{-/-}$ brain stems were found to have a statistically significant increase in viral replication as compared to wild-type controls following corneal challenge (Fig. 3.4B). Although the differences were not statistically significant, whole brain titers from IRF-3$^{-/-}$ mice were also increased compared to control (Fig. 3.4C). Together, these data suggest that IRF-3$^{-/-}$ mice have a deficit in their ability to control lethal brain infection.

Loss of IRF-3 results in increased viral replication and increased mortality following intracranial HSV-1 infection. The cornea model of HSV-1 infection mimics the physiological course of eye disease seen in humans and permits evaluation of viral replication in peripheral tissues. However, many factors can affect the ability of the virus to replicate in peripheral tissues and also affect its ability to enter the brain and replicate therein. Therefore, to evaluate the role of IRF-3 on HSV replication in the brain directly, 100 PFU HSV-1 strain 17 was inoculated into the cortex and the mice were evaluated for mortality and viral replication. There was a significant increase in lethality of HSV in the IRF-3$^{-/-}$ mice as compared to the controls (Fig 3.5A). While over 60% of the WT mice survived IC injection, less than 20% of the IRF-3$^{-/-}$ mice survived the same challenge. Correspondingly, beginning at day 3 and continuing at day 5, IRF-3$^{-/-}$ brains permitted a 10-100 fold increase in viral replication as compared to control mice (Fig. 3.5B). These

results suggest that IRF-3-mediated pathways are important in controlling HSV-1 replication in brain tissues following direct intracranial injection.

IRF-3$^{-/-}$ mice had increased and altered antigen staining following IC challenge with HSV-1. WT and IRF-3$^{-/-}$ mice were infected intracranially with 100 PFU HSV-1 strain 17 and harvested at day 3 and 5 post infection. Sagittal sections of the brain were stained with an anti-HSV-1 polyclonal antibody. Sections were divided into five regions (olfactory bulb, central brain, mid-brain cerebellum, and pons/medulla/brain stem), and scored as either positive or negative for HSV antigen staining in a masked fashion. Following scoring, total antigen positive regions were then divided by total sections counted in order to calculate a percentage of antigen-positive regions (Table 1, Fig 3.6-7). In general, IRF-3$^{-/-}$ mice displayed a higher percentage of antigen-positive regions than WT mice. The central brain region (cerebral cortex, hippocampus, septum, thalamus, and hypothalamus) was the site of inoculation and displayed a consistent and high percentage of antigen-positive regions at both day 3 and 5 in both mice (Figure 3.6). In contrast, the mid-brain, cerebellum, and brain stem displayed little antigen staining (<10%) in either WT or IRF-3$^{-/-}$ mice at day 3 (Fig. 3.6A). By day 5, however, IRF-3$^{-/-}$ mice displayed a significant increase in antigen positive sections as compared to controls in midbrain, cerebellum and brain stem (Fig. 3.6A). In addition to increased antigen positive regions, IRF-3-/- mice displayed a distinct antigen staining pattern compared to WT mice as shown in representative images from the central brain. HSV staining of WT lesions showed staining foci in cells with neuronal morphology (3.6B). In contrast, IRF-3-/- brain sections had generalized antigen positive lesions with entire areas appearing

uniformly stained with no apparent foci (Fig. 3.6C). This altered staining was consistent in each IRF-3$^{-/-}$ antigen positive region examined and suggest increased antigen production and spread in IRF-3$^{-/-}$ brains compared to control (Fig. 3.7). Together, the increase in antigen production and distribution correlate with the previously observed increased viral titers in IRF-3$^{-/-}$ brains (Fig. 3.4 & 3.5).

IRF-3$^{-/-}$ mice produce increased amounts of inflammatory cytokines following direct intracranial infection. In addition to HSV-1 replication, several studies have implicated inflammatory cytokines as contributing to increased lethality following CNS infection (31-32). To assess inflammatory cytokine production in IRF-3$^{-/-}$ mice, total brain homogenates were prepared and cytokines were assayed by a bead-based multiplex array following IC infection with 100 PFU of HSV-1 strain 17 or mock treatment (Fig. 3.8). In both the IRF-3$^{-/-}$ and WT brains, cytokine samples taken on day 3 were showed minimal induction of cytokines with little or no variation between the virus-infected or mock-treated groups. At day 5, however, there was a significant increase in several inflammatory cytokines in infected IRF-3$^{-/-}$ brains as compared to WT infected mice. IRF-3$^{-/-}$ brains produced a 3.5 fold increase in IL-1β, a 4.6 fold increase in TNFα, and a 5.8 fold increase in IL-6 as compared to infected WT brains. This trend also extended to IL-12 (7.1 fold), IL-10 (3.6 fold), as well as several chemokines including MCP-1, Rantes, and MIP1β. In contrast, one cytokine (KC) demonstrated similar levels of production in the WT and IRF-3$^{-/-}$ group. In addition, several were globally upregulated (IL-5, IL-13, GM-CSF) in both WT and IRF-3$^{-/-}$ mice following virus- or mock-infection suggesting that mechanical damage of injection was sufficient to induce their expression

(Fig 3.8, data not shown). Together, the data suggest that in response to HSV-1 infection, IRF-3$^{-/-}$ deficient mice produce a stronger inflammatory response as measured by cytokine production. The timing of this increase in inflammatory cytokines, on day 5, also coincided with the lethality seen in this model of infection.

IRF-3$^{-/-}$ mice have increased cytokine expression in the brain stem following peripheral infection. Having shown increased cytokine expression in brains following direct intracranial injection, it was of interest to observe changes in cytokine levels following peripheral infection. Examination of the brain stem revealed increased production for several cytokines in the IRF-3$^{-/-}$ mice as compared to control mice (Fig. 3.9). While maintaining similar levels at day 3, several cytokines had increased expression at both days 5 and 7 in IRF-3-deficient mice. For example, there was increased expression of IL-6, IL-12 and IFNγ at days 5 and 7 post infection. Other cytokines had increased only at day 7 including IL-10, MCP-1, and G-CSF (data not shown). The increased cytokine production at this late time corresponded with the peak in lethality seen following corneal infection, consistent with inflammation being a cause of increased mortality in IRF-3$^{-/-}$ mice.

IRF-3 deficient mice have a deficiency in type I IFN production. Previous work demonstrated a deficit in the production of IFNβ following infection of IRF-3$^{-/-}$ bone marrow-derived dendritic cells as compared to control cells (40). The current experiments sought to determine whether IRF-3-deficient mice displayed a similar IFNβ production deficit in brain tissues following IC infection. WT and IRF-3$^{-/-}$ mice were challenged with

a high dose of HSV-1 and brains were harvested 12 and 18 hours post infection. At both 12 and 18 hours post infection there was a statistically significant difference in IFNβ protein levels in IRF-3$^{-/-}$ mice as compared to WT control mice (Fig. 3.10A). At 12 hours post infection, WT mice produce nearly 3.5 fold more IFNβ compared to IRF-3$^{-/-}$, and 2.2 fold more IFNβ at 18 hours post infection. The 12 and 18 hour results therefore recapitulated the results previously reported for BMDCs(40) and are consistent with the observation that IRF-3$^{-/-}$ mice permit increased viral replication, and show increased susceptibility to infection.

Previous studies *in vivo* showed no change in serum IFNα levels relative to control mice following intravenous infection of IRF-3$^{-/-}$ mice with HSV-1 (21). The authors concluded that IRF-7 was primarily responsible for IFNα production *in vivo.* To further assess that idea in this work, brain samples were assayed by ELISA for IFNα (Fig. 3.10B). The data showed a defect in IFNα production in IRF-3$^{-/-}$ mice as compared to WT control mice at all time points tested. At 12 hours post infection, WT mice have nearly 5 fold more IFNα than IRF-3- deficient brains which remained at minimal levels. However, by 18 hours, IFNα production from IRF-3$^{-/-}$ brains was significantly above background levels suggesting an IRF-3 independent response to viral challenge, consistent with a role for IRF-7. While this IFNα production is still notably deficient compared to WT, it does suggests that IRF-3$^{-/-}$ mice are capable of inducing the type I IFN cascade. Together, the results confirm a deficit and delay in the induction of type I IFN in IRF-3$^{-/-}$ mice *in vivo.*

The previous results demonstrated a significant difference between IRF-3$^{-/-}$ and WT mice in terms of type I IFN production. However, those experiments required a higher dose of HSV-1; attempts at a lower dose had failed to detect IFN within the linear range of the ELISA assay. Therefore, following IC infection with 100 PFU of HSV-1, WT and IRF-3$^{-/-}$ brains were removed, the olfactory bulb and brain stem discarded, and RNA harvested from the remaining brain for analysis 18 hours post infection. The results demonstrated a statistically significant decrease in fold expression of IFNβ RNA in IRF-3$^{-/-}$ mice as compared to controls (Fig. 3.10C). The WT brains averaged a 3.4 fold increase in IFNβ transcript as compared to mock samples; while in contrast, IRF-3$^{-/-}$ brains averaged a 1.9 fold increase. These results, coupled with the type I IFN ELISA results following high dose infection, demonstrate a deficiency in type I IFN production in the brains of IRF-3$^{-/-}$ mice.

Discussion

The data in this study show that IRF-3 plays a critical role in the control of HSV-1 CNS infection. While no impact of loss of IRF-3 was observed in peripheral tissues, its absence significantly delayed and reduced type I IFN production in the brain. Therefore, deficient IFN response most likely permitted HSV-1 to establish a foothold for infection, resulting in increased viral replication and antigen staining in IRF-$3^{-/-}$ brains. Concomitant with increased viral replication, the immune system induced an increased inflammatory cytokine response in IRF-3-deficient mice and these factors combined to result in significantly increased lethality. Together, these results highlight the importance of the IRF-3 dependent immune response in preventing lethal CNS infection following HSV-1 challenge.

Similar to previous reports in immune cells (40), IRF-$3^{-/-}$ deficient mice had reduced type I IFN production in the brain following HSV-1 challenge, and delayed or reduced type I IFN production has broad implications for susceptibility to viral replication in the CNS. Deficient type I IFN production in brains contributes to encephalitis in a variety of RNA virus infections including West Nile, Semliki virus, and mouse hepatitis virus (8-9, 15, 22). The results also correspond with genetic studies in humans demonstrating a deficiency in TLR signaling, specifically TLR-3, whose loss results in increased susceptibility to HSE (5, 68). While TLR3 is dispensable for protection of mice from viral infection, IRF-3 is apparently required for protection. IRF-3 is downstream of TLR-3 in the signaling pathway and these findings emphasize the importance of this type I IFN induction pathway in controlling HSE *in vivo* (59).

In addition to controlling viral replication, the inflammatory response to CNS infection is also thought to contribute to lethality following HSV-1 challenge. Indeed, the inflammatory response is both protective and harmful to the host during HSE. Deletion or inhibition of parts of the inflammatory response result in the host succumbing to HSV-1 infection (4, 34, 55). In contrast, antagonizing other inflammatory elements has positive results in terms of morbidity and mortality (31-32, 41). In the absence of type I IFN signaling, several viruses have been reported to induce increased CNS inflammation in addition to increased viral replication (22, 57). A similar pattern emerges in these studies, as IRF-3$^{-/-}$ mice have increased inflammatory cytokine production in the brains following IC and cornea infection. The increase in inflammatory cytokine production in IRF-3$^{-/-}$ mice preceded the major peak in lethality in both models. These data suggest that increased inflammatory cytokine production, in addition to increased viral replication, result in the increased mortality seen in IRF-3$^{-/-}$ mice.

Previous work with other viruses has suggested an alteration in viral distribution or viral tropism in the context of defective or antagonized type I IFN signaling (16, 22, 51). In this study, assessment of viral antigen distribution revealed that while initially limited to the central brain region, HSV-1 was distributed in the brain stem, cerebellum, and mid-brain in both WT and IRF-3$^{-/-}$ mice by day 5 following IC infection. In each region, IRF-3$^{-/-}$ brains exhibited a higher percentage of antigen positive regions, but the overall location of the virus was similar between the WT and IRF-3$^{-/-}$ mice. There was, however, a distinct antigen staining patterns in IRF-3$^{-/-}$ and WT brain sections. IRF-3$^{-/-}$ mice showed lesions with uniform antigen positive regions while WT lesions showed HSV-1 staining foci in cells with neuronal morphology. This observation is consistent

with the hypothesis that IRF-3$^{-/-}$ mice permitting initial uncontrolled viral replication and resulting in wide, uniform antigen staining.

The data presented in this study demonstrate a more complex role for IRF-3 than previously shown (21). There is consistency between the previous study and the current data examining replication in corneas, trigeminal ganglia, and periocular skin (data not shown), but there are also some sharp distinctions when considering the current observation of increased lethality and brain titers. A possible explanation is the nature of the immune response in the CNS. In peripheral tissues, the type I IFN response is primarily driven by plasmacytoid dendritic cells in an IRF-7 dependent manner; high levels of IFNα are produced which can compensate for the loss of IRF-3 dependent pathways (27). This model is supported by the previous intravenous challenge data (21) and data in this study. In contrast, CNS tissues require local production of type I IFN to control viral infection; serum IFN cannot compensate for an IRF-3 deficiency because peripheral type I IFN fails to penetrate the blood brain barrier (10-11). Therefore, in the brains of IRF-3$^{-/-}$ mice, HSV-1 can replicate uninhibited for several hours without hindrance from type I IFN. Eventually, the type I IFN response is triggered through an IRF-3 independent pathway, but by this time HSV-1 has established a foothold in the CNS. This facilitates increased viral titers and inflammatory cytokine production in IRF-3$^{-/-}$ mice and lead to encephalitis and a significant increase in lethality.

Taken together, these data demonstrate a critical role for IRF-3 in the brain following HSV-1 challenge. The results also demonstrate a major delineation between the peripheral and CNS innate immune responses. The data also underscore the importance of testing multiple infection models, and measuring multiple parameters to

fully ascertain the roles of host resistance factors in viral infection. Ongoing experiments in our laboratory seek to evaluate changes in viral tropism and inflammatory infiltrates in the brain of IRF-$3^{-/-}$ mice. Further experiments will determine the precise pathways and molecules responsible for HSV-1 recognition. Several candidates involved in the early recognition pathways have been implicated and cells and mice lacking these components are being evaluated both *in vitro* and *in vivo*.

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Figure 3.1. IRF-3-/- mice have no change in peripheral tissues following infection with HSV-1 17. WT and IRF-$3^{-/-}$ mice were infected with $2x10^6$ pfu HSV-1 17 per eye. (A) Cornea swabs, (B) periocular skin, and (C) trigeminal ganglia were harvested and titered at the specified days. The graphs represent the average of several mice at each time point from two independent experiments.

Figure 3.2. Reactivation is not changed in IRF-3-/- mice as compared to control. WT and IRF-$3^{-/-}$ mice were infected with $2x10^6$ pfu HSV-1 17 per eye. Following establishment of a latent infection, defined as 28 days post-infection, mice were sacrificed and trigeminal ganglia (TGs) harvested. TGs were bisected and co-cultured on a monolayer of Vero cells. Supernatants were removed daily for 7 days post-explant and added to fresh Vero monolayers. This monolayer was then scored for cytopathic effect and the results were recorded as the percentage of wells positive for reactivation.

Figure 3.3. IRF-3 plays a minimal role in peripheral tissues following corneal infection with HSV-1 McKrae. WT and IRF-$3^{-/-}$ mice were infected with $2x10^6$ pfu HSV-1 McKrae per eye. (A) Cornea swabs and (B) trigeminal ganglia were harvested and titered at the specified days. The graphs represent the average of several mice at each time point from two independent experiments.

Figure 3.4, IRF-$3^{-/-}$ mice have increased lethality and increased viral replication in brain tissues following HSV-1 McKrae cornea infection.. (A) Survival plot following infection of WT and IRF-$3^{-/-}$ deficient mice with $2x10^6$ pfu HSV-1 McKrae per eye.

Survival experiments were conducted independently of the other experiments and represent the sum of multiple experiments. (B) Brains and (C) brain stems were harvested and titered at the specified days following infection of WT and IRF-$3^{-/-}$ deficient mice with $2x10^6$ pfu HSV-1 McKrae per eye. The graphs represent the average of several mice from two independent experiments. The dotted line represents the limit of detection for this assay. **p value <0.01.

Figure 3.5. IRF-$3^{-/-}$ mice have reduced survival and increased viral titers in the brain following HSV-1 strain 17 intracranial infection.. (A) Survival plot of IRF$3^{-/-}$ and WT mice following intracranial infection with 100 pfu HSV-1 17. Survival experiments were conducted independent of the other experiments and represents the sum of experiments. (B) Viral titers in whole brain tissue harvested at the specified days. Data represents the average of several mice from two independent experiments. The dotted line represents the limit of detection for this assay. *p value <0.05 **p value <0.01.

Figure 3.6. IRF-$3^{-/-}$ brain sections have increased antigen stain following intracranial HSV-1 infection. Following IC infection with 100 PFU HSV-1 strain 17, brains were harvested on day 3 and day 5 post infection, formalin fixed, sectioned sagittally, and stained with a polyclonal anti-HSV antibody. Sections were divided into five regions (olfactory bulb, central brain, mid-brain cerebellum, and pons/medulla/brain stem), and scored as either positive or negative for HSV antigen staining in a masked fashion. Following scoring, total antigen positive regions were then divided by total

sections counted in order to calculate a percentage of antigen-positive regions for day and day 5 (A). B and C, representative immunoperoxidase stained (?) images from the central brain region of WT and IRF-3$^{-/-}$ mice . *p value <0.05 **p value <0.01

Table 3.1. Summary of antigen scoring in IRF-3-/- and WT mice following IC infection with HSV-1 or mock. Following IC infection with 100 PFU HSV-1 strain 17 or mock, brains were harvested on day 1, day 3, and day 5 post infection, formalin fixed, sectioned sagittally, and stained with a polyclonal anti-HSV antibody. Sections were divided into five regions (olfactory bulb, central brain, mid-brain cerebellum, and pons/medulla/brain stem), and scored as either positive or negative for HSV antigen staining in a masked fashion. Following scoring, total antigen positive regions were then divided by total sections counted in order to calculate a percentage of antigen-positive regions for each day and condition.

Figure 3.7. Representative brain sections from IRF-3-/- following HSV-1 infection demonstrate altered antigen staining compared to control. Following IC infection with 100PFU HSV-1 strain 17, brains were harvested on day 5, formalin fixed, sectioned sagittally, and stained with a polyclonal anti-HSV antibody. Representative immunoperoxidase stained images of mock, WT, and IRF-3-/- from the (A) (B) (C) regions are shown.

Figure 3.8. IRF-3$^{-/-}$ mice show increased inflammatory cytokine production following intracranial infection with HSV-1 strain 17. Following IC infection with

100 PFU HSV-1 17, brains were harvested on days 3 and 5 post infection, processed, and assayed via a bead based cytokine assay (BioPlex, Bio-Rad). The results shown are the average 4-6 mice brain stems per group per time point. Statistical calculations based on infected WT and infected IRF-$3^{-/-}$ mice. *p value <0.05.

Figure 3.9. IRF-$3^{-/-}$ mice show increased inflammatory cytokine production following peripheral infection. Following IC infection with 100 PFU HSV-1 17, brains were harvested on days 3 and 5 post infection, processed, and assayed via a bead based cytokine assay (BioPlex, Bio-Rad). The results shown are the average 4-6 mice brain stems per group per time point. *p value <0.05 **p value <0.01***p value<0.001.

Figure 3.10. IRF-$3^{-/-}$ mice have a deficit in type I IFN production following intracranial infection with HSV-1. WT and IRF-$3^{-/-}$ deficient mice were infected with $1x10^6$ pfu HSV-1 strain 17. Whole brain tissue was harvested as specified times, processed, and analyzed for (A) IFNβ and (B) IFNα by ELISA (PBL Laboratories). Results shown represent the average of 10-14 mice per group per time point from two separate experiments. (C) Following infection with 100pfu HSV-1 strain 17, brain tissue, excluding brain stem and olfactory bulb, were harvested for RNA 18 hours post infection. Samples were assayed by real-time RT-PCR and are expressed as fold expression over mock infected samples. Results shown are the average fold expression from 6-7 mice per group per time point from two separate experiments. *p value <0.05 **p value <0.01***p value<0.001.

Figure 3.1. IRF-3$^{-/-}$ mice have no change in peripheral tissues following infection with HSV-1 17.

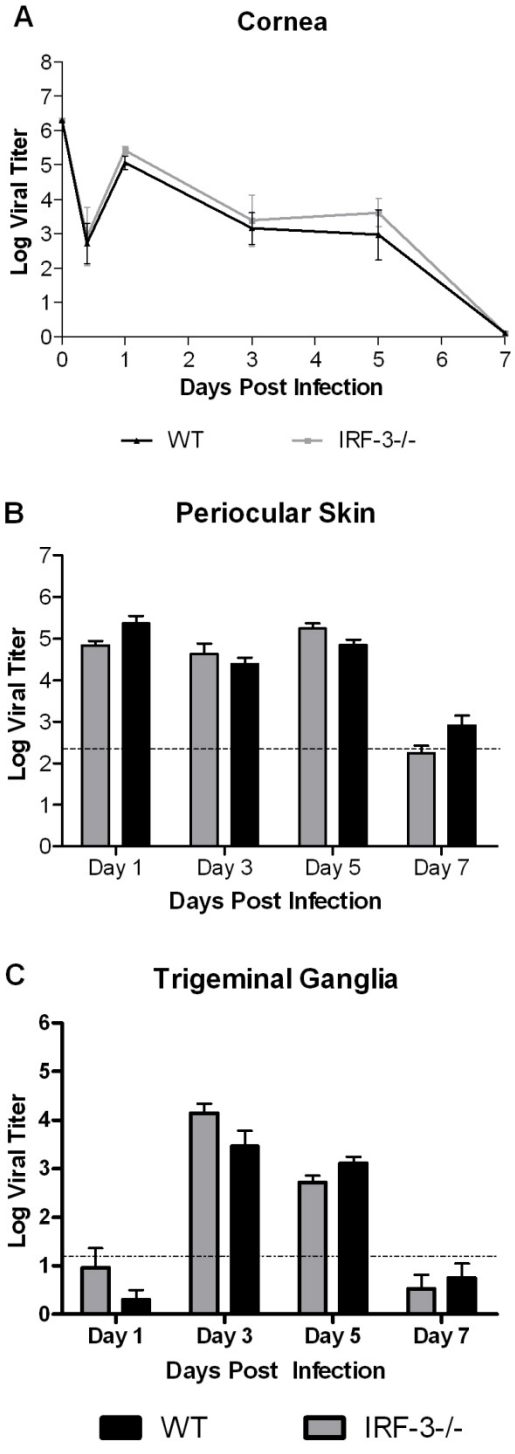


Figure 3.2. Reactivation is not changed in IRF-3$^{-/-}$ mice as compared to control.

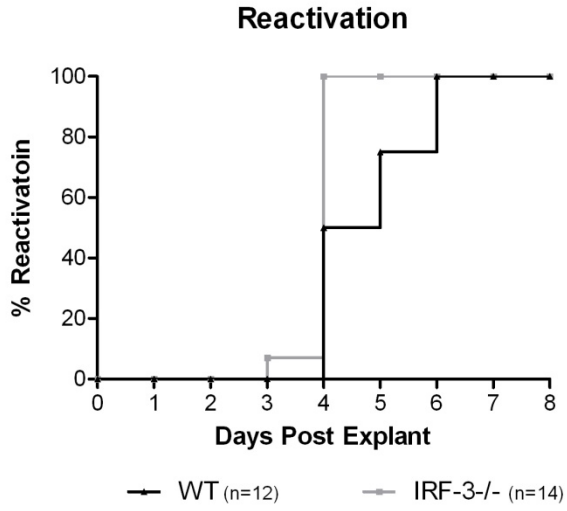


Figure 3.3. IRF-3 plays a minimal role in peripheral tissues following corneal infection with HSV-1 McKrae.

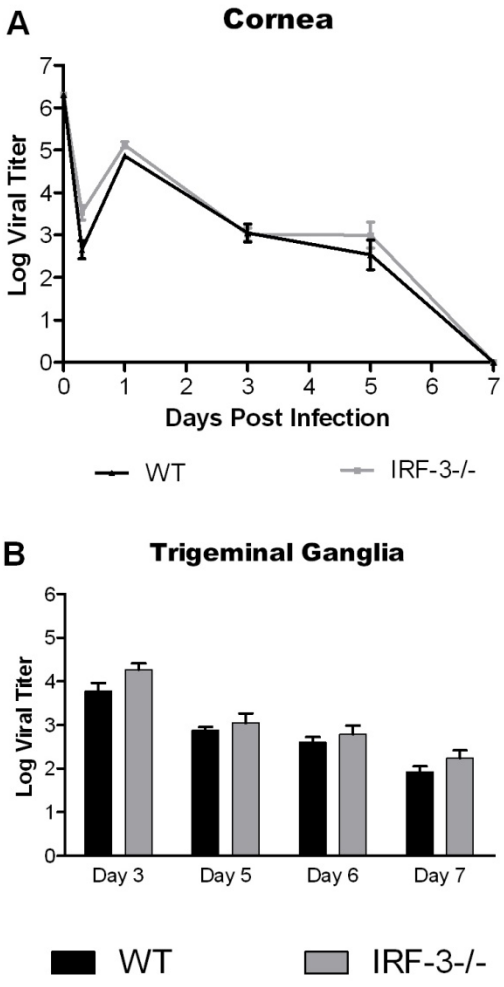


Figure 3.4, IRF-3$^{-/-}$ mice have increased lethality and increased viral replication in brain tissues following HSV-1 McKrae cornea infection..

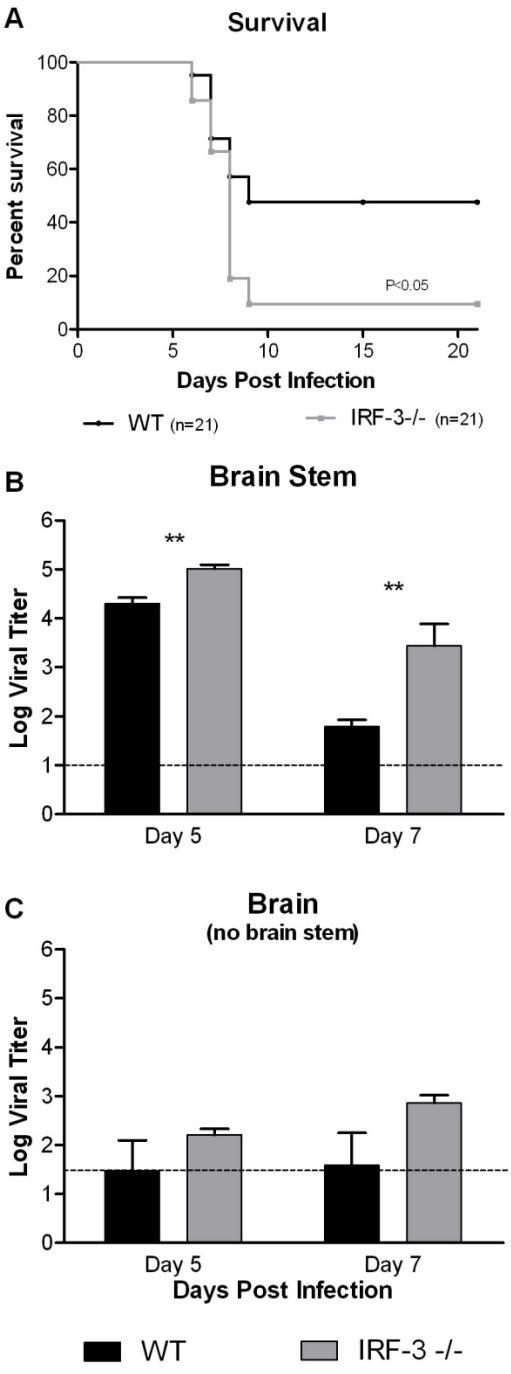


Figure 3.5. IRF-3$^{-/-}$ mice have reduced survival and increased viral titers in the brain following HSV-1 strain 17 intracranial infection.

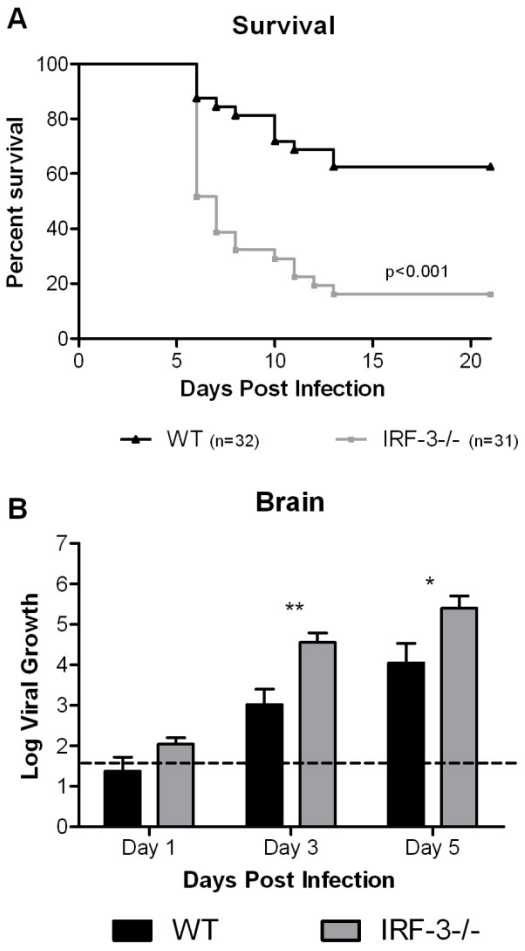
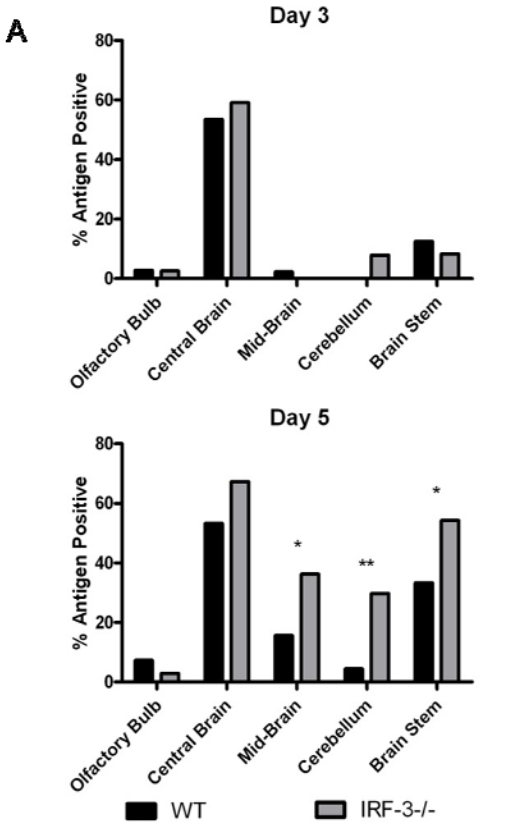
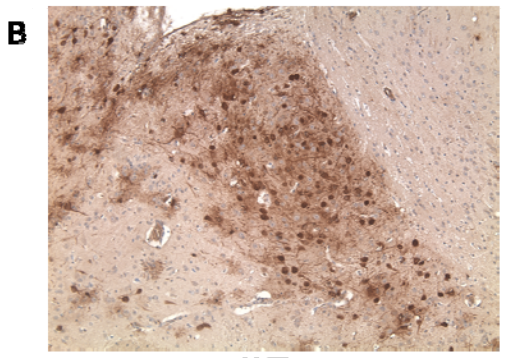


Figure 3.6. IRF-3$^{-/-}$ brain sections have increased antigen stain following intracranial infection.





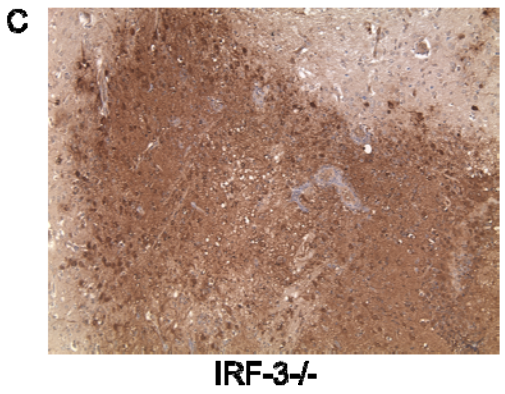


Table 3.1, Summary of antigen scoring in IRF-3$^{-/-}$ and WT mice following IC infection with HSV-1 or mock.

Sample	Olfactory Bulb	Central Brain	Mid-Brain	Cerebellum	Brain Stem	Number of Mice
IRF-3$^{-/-}$ Day 3	2.6% (1/39)	59.1% (26/44)	0% (0/42)	7.9% (3/38)	8.3% (3/36)	9
WT Day 3	2.9% 1(35)	53.5% (23/43)	2.3% (1/43)	0% (0/38)	12.5% (5/40)	8
IRF-3-/- Day 5	2.9% (1/34)	67.3% (33/49)	36.2% (17/47)	29.8% (14/47)	54.2% (26/48)	9
WT Day 5	7.3% (2/41)	53.5% (24/45)	15.6% (7/45)	4.4% (2/45)	33.3% (14/42)	9
IRF-3-/- Mock	0% (0/16)	6.3% (1/16)	0% (0/16)	0% (0/15)	0% (0/16)	4
WT Mock	0% (0/9)	8.3% (1/12)	0% (0/12)	0% (0/12)	0% (0/12)	3

Figure 3.7, Representative brain sections from IRF-3$^{-/-}$ following HSV-1 infection demonstrate altered antigen staining compared to control.

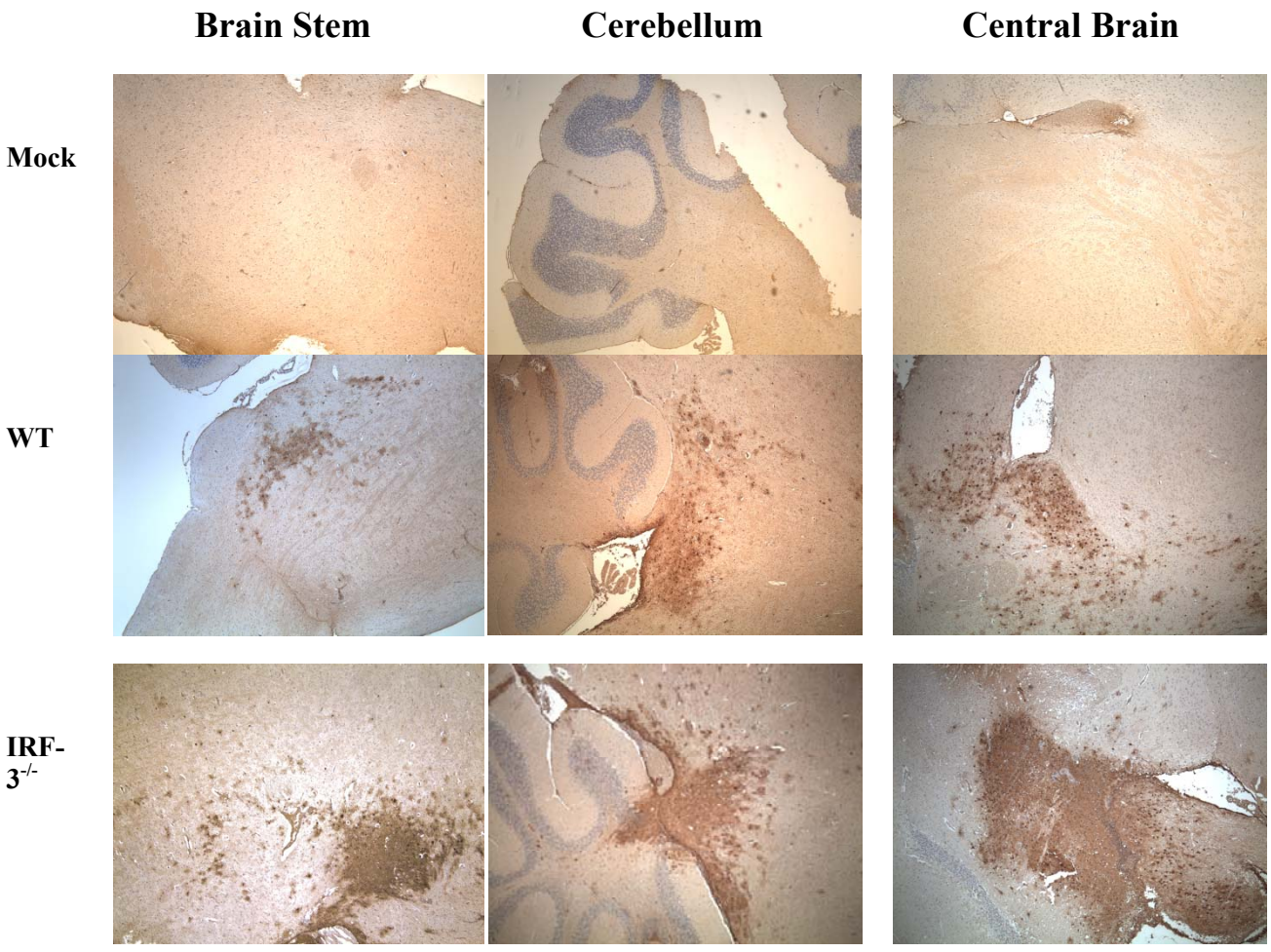


Figure 3.8. IRF-3$^{-/-}$ mice show increased inflammatory cytokine production following intracranial infection.

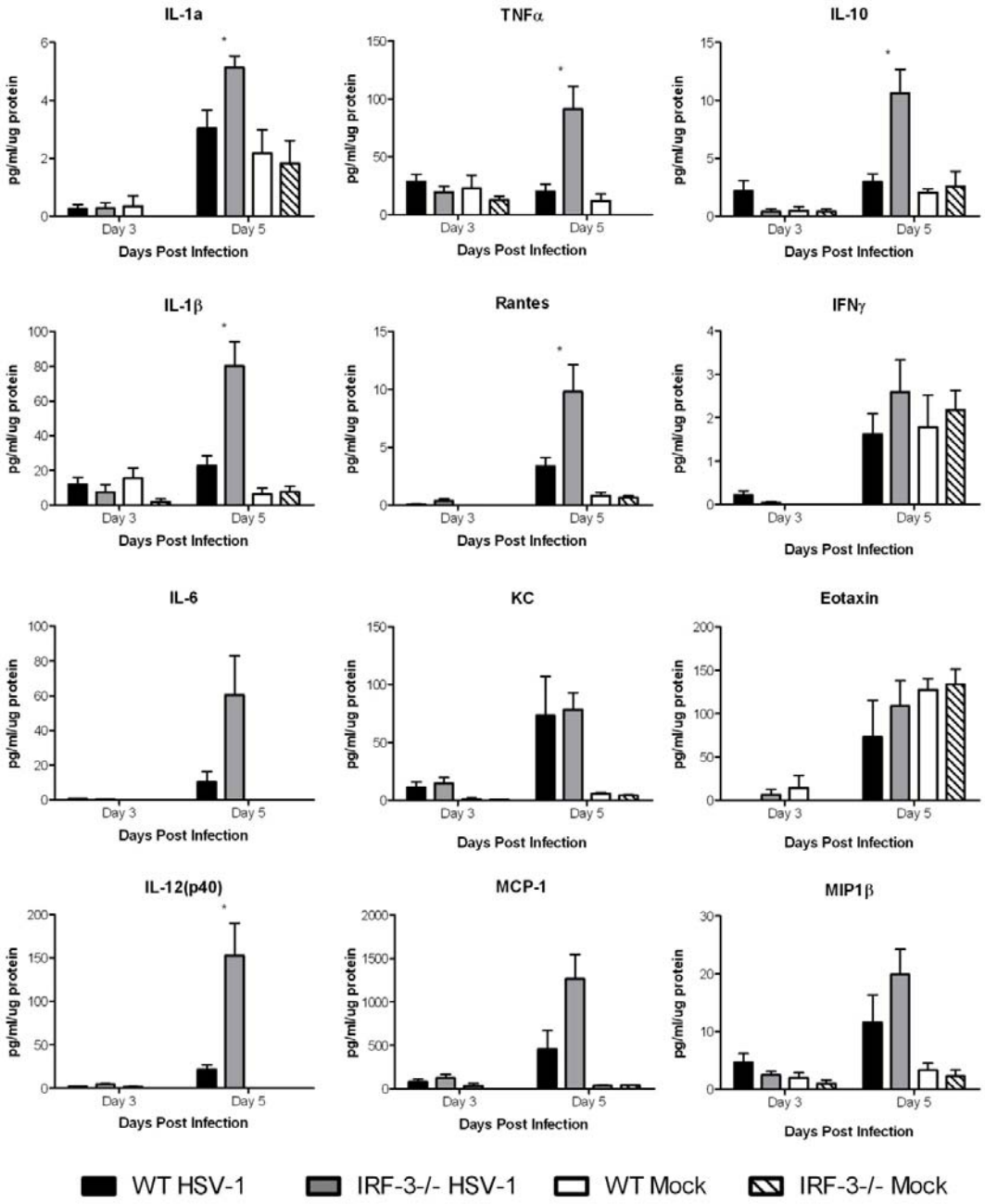


Figure 3.9. IRF-3$^{-/-}$ mice show increased inflammatory cytokine production following peripheral infection.

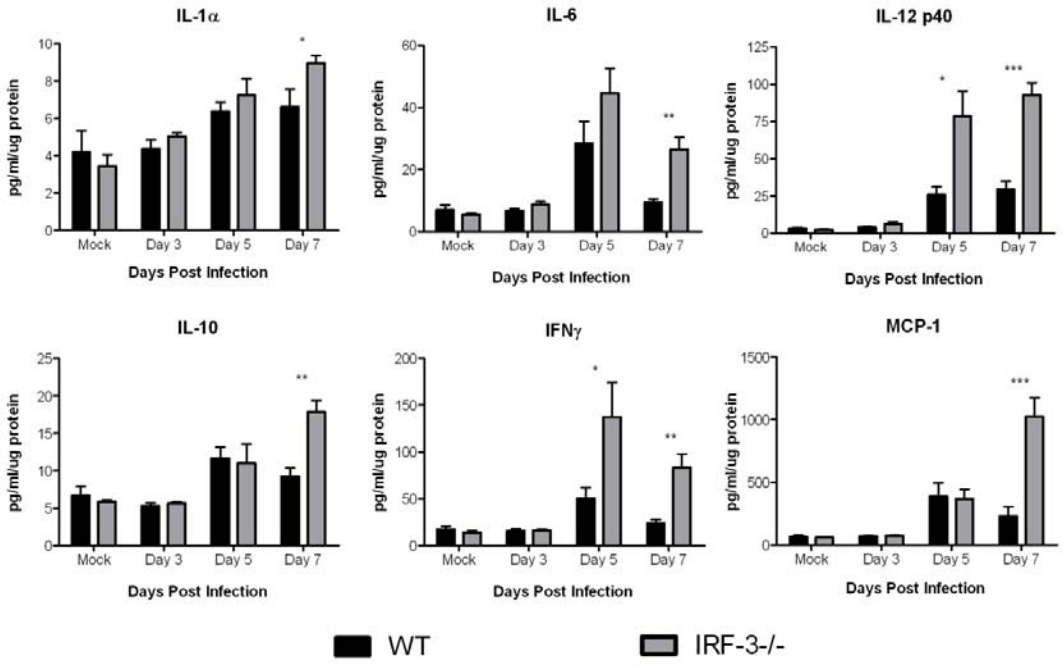
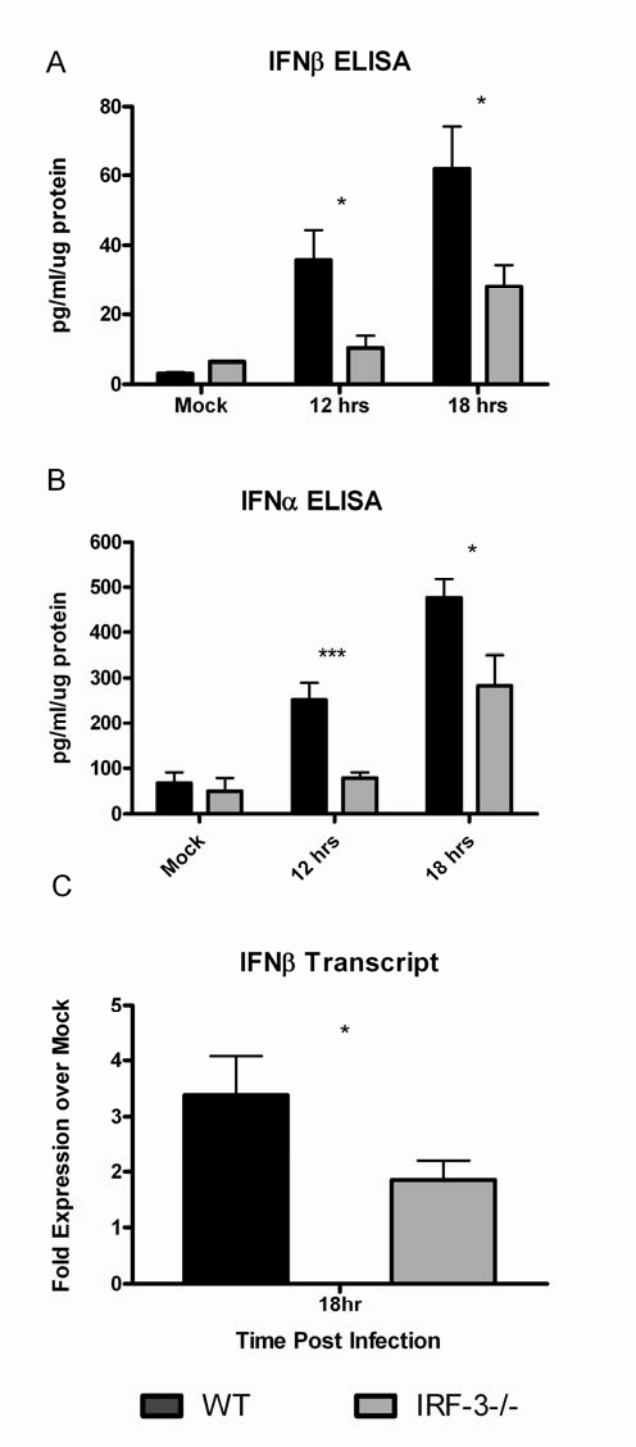


Figure 3.10. IRF-3$^{-/-}$ mice have a deficit in type I IFN production following intracranial infection with HSV-1.



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Chapter IV

Summary, Implications, and Future Directions

Summary of Research and Goals

The major goal of this research was to address the role of interferon regulatory factor-3 (IRF-3) on herpes simplex virus type 1 (HSV-1) infection both *in vitro* and *in vivo*. Prior to these studies, examination of IRF-3 in the context of HSV-1 infection had led to contradictory results. Several studies argued that IRF-3 dependent pathways had minimal impact on HSV-1 infection *in vitro* or *in vivo* (5, 14). In contrast, other studies demonstrated that HSV-1encodes viral proteins that both directly and indirectly target IRF-3 activity, implying a role for IRF-3 in the immune response to HSV-1 (12, 17, 23-26, 31, 33). Therefore, the studies in this thesis sought to address the contradiction in these data and clarify a role for IRF-3 in the control of HSV-1 infection.

While numerous studies have implicated the type I IFN response in control of virus infection, the data presented here advocates a critical role for IRF-3 and the early recognition pathways in initiating control of HSV-1 *in vitro* and *in vivo*. In the absence of IRF-3, immune cells *in vitro* and brain tissues *in vivo* demonstrated a deficit in type I IFN production following HSV-1 challenge. In each case, the IRF-$3^{-/-}$ cells and animals eventually produced a type I IFN response; however, the delay and reduction in type I IFN early during infection permitted an immediate increase in HSV-1 replication. Over the course of infection, the small change in IFN production early during infection manifested in enhanced viral replication leading to an increase in cytokine production. These factors led to greater lethality for IRF-$3^{-/-}$ mice *in vivo*; therefore, the absence of IRF-3 resulted in a significant shift in susceptibility.

However, contrasting major type I IFN cascade knockouts like STAT-$1^{-/-}$ and IFN$\alpha\gamma R^{-/-}$, IRF-3 deficiency resulted in no temporal shift in the survival curve, but only a shift in total susceptibility. WT and IRF-$3^{-/-}$ mice died with the same kinetics after both

ocular or intracranial HSV-1 infection; the major delineation was the total percentage of dead. The data suggest that the loss of IRF-3 simply increased immediate HSV-1 replication creating an infectious dose that mimics a higher viral inoculation. The result was increased lethality in IRF-$3^{-/-}$ mice, but in both mice, the likely mechanisms causing death were a combination of viral replication and subsequent inflammation in the CNS. While the loss of IRF-3 does not ablate a type I IFN response, the delay in induction results in an early increase in HSV-1 replication which has major impact on the course and outcome of infection. Overall, the loss of IRF-3 demonstrates the importance of the early recognition pathways on a timely immune response to viral challenge.

From *in vitro* studies, the necessity of IRF-3 appears to be cell-type dependent. In MEFs, the loss of IRF-3 has minimal impact on HSV-1 replication (14). In contrast, bone marrow derived dendritic cells and macrophages yield increased HSV-1 titers in the absence of IRF-3. One explanation implicates the differing responses to virus infection in these cell types. Immune cells like BMDCs and BMM are expected to have a vigorous immune response to viral challenge, producing and responding to type I IFN production. In contrast, while MEFs respond to type I IFN stimulation, the cells are unable to completely control HSV-1 replication even with IFN pretreatment. In addition, loss of critical components of the IFN response including STAT-1 or IFNαR has only modest impact on HSV-1 replication in MEFs. These data suggest that either an insufficient immune response, HSV-1 antagonism, or a combination of both result in reduced efficacy of type I IFN in MEFs. In immune cells, the opposite may be true with a sufficient immune response resulting in less effective HSV-1 antagonism. The loss of IRF-3 in this situation results in increased HSV-1 replication in these cell types. Together, the data

demonstrate the importance of cell type selection to *in vitro* experiments. While MEFs represent a standard cell type, their use in examination of the immune system may be inadequate. At a minimum, immune cells like BMDCs and BMM should be used in addition to MEFs in characterization of immune functions.

In vivo, the necessity of IRF-3 appeared to be CNS specific. Loss of IRF-3 in peripheral tissues has minimal impact on viral replication following HSV-1 challenge. However, in the brain, IRF-3 deficiency resulted in enhanced viral replication and greater inflammatory cytokine titers which likely account for increased lethality seen in IRF-$3^{-/-}$ mice. Examination of type I IFN production revealed a deficit in IRF-$3^{-/-}$ brains as compared to controls, providing a mechanism for increased HSV-1 replication and suggesting a CNS specific necessity for IRF-3. While peripheral tissues primarily utilize type I IFN production by plasmacytoid dendritic cells (pDCs), the brain is largely devoid of this cell type (1-2, 30). Instead, the CNS relies on resident cells to produce and respond to type I IFN (9). In the absence of IRF-3, the CNS fails to produce an immediate type I IFN response and HSV-1 establishes a foothold for infection. Augmented viral replication follows which leads to increased cytokine production and increased lethality. In the periphery, the loss of IRF-3 impacts local production of type I IFN as demonstrated by reduced IFNβ (14, 29). However, infiltration by immune cells and IRF-7 mediated production of IFNα likely rescues the type I IFN cascade and prevents the virus from establishing a foothold in peripheral tissues. This exogenous IFN production by infiltrating cells is not available in the CNS as few pDCs are found in the brain and type I IFN has not been shown to pass through the blood brain barrier (9-10). Therefore, the CNS requires local production of type I IFN and IRF-3 is critical for a

timely and efficient response. In the absence of IRF-3, the virus gains its foothold and the result is increased susceptibility to HSV-1 CNS infection.

Implication of Thesis

Several studies have demonstrated the importance of type I IFN in limiting viral infection in the periphery and the CNS. In the absence of IFNα/γR or IFN signaling, mice become very susceptible to infection caused by a variety of viruses including HSV-1 (6, 8, 13, 15-16, 19). In this study, the loss of IRF-3 also renders mice more susceptible to HSV-1 infection and lethality, but only in the context of CNS infection. The results contrast reports from West Nile virus (WNV) infection of IRF-$3^{-/-}$ mice. Deficiency in IRF-3 results in increased WNV replication in peripheral tissues, altered tissue tropism, and earlier entry into the CNS (6). This data demonstrate that in the context of WNV infection, IRF-3 is an essential regulator in both peripheral and CNS tissues. However, the loss of IRF-3 has no detectable impact on HSV-1 replication in the periphery.

One possible explanation is increased sensitivity of HSV-1 to type I IFN as compared to WNV. In the periphery, the loss of IRF-$3^{-/-}$ has been shown to delay, but not significantly diminish systemic accumulation of type I IFN in response to WNV or HSV-1 infection (6, 14). The delay in type I production potentially permits a small, immediate increase in viral replication in both virus types. However, the eventual type I IFN response in the periphery serves to limit HSV-1 infection and spread. In contrast, WNV is relatively resistant to type I IFN and capitalizes on the initial delay with increased viral replication. Another possible explanation involves cell-type tropism; WNV infects a wider range of cells during its normal course of infection and thus requires the ability to

modulate the immune response in a variety of cell types. In contrast, HSV-1 primarily infects epithelial cells and neuronal cell types during physiologically infections; it bypasses the need to infect and control immune cells by transport via neuronal axons. Therefore, HSV-1 may not have developed methods to antagonize the immune response in these cell types.

Immune cells, including BMDCs and BMM, require IRF-3 dependent pathways to limit HSV-1 replication. In the absence of IRF-3, both BMDCs and BMMs are unable to control HSV-1 replication until type I IFN is produced via an alternate pathway. This results in control of HSV-1 infection once the type I IFN response is initiated in the periphery *in vivo*. This hypothesis is supported by studies of STAT-$1^{-/-}$ and IFNαβγ$R^{-/-}$ mice. Loss of type I and type II IFN receptors results in multi-organ failure and systemic infection following HSV-1 challenge (19). In contrast, STAT-$1^{-/-}$ mice control peripheral viral replication, but succumb to CNS infection following HSV-1 infection. A major distinction between these mice was the ability to produce and respond to type I IFN via the type I IFN receptor (unpublished, Pasieka et al). Antibody blockade of IFNαR resulted in systemic infection and organ failure in STAT-$1^{-/-}$ mice. Together, the data from the IRF-$3^{-/-}$ and STAT-$1^{-/-}$ mice demonstrate that systemic HSV-1 infection is severely limited by even an attenuated type I IFN response in the periphery.

In contrast to the periphery, the data demonstrates that IRF-3 dependent pathways are critical in limiting viral replication and lethality following infection of the CNS. In both HSV-1 and WNV, the loss of IRF-3 results in increased HSV-1 replication in the CNS tissues following direct and peripheral infection (6). These data correlate with observations in humans regarding the role of TLR pathways in herpes simplex

encephalitis (HSE). Inborn disorders of type I IFN production have been demonstrated to render healthy individuals susceptible to HSE, most notably mutation in TLR-3 (3, 35). In addition, TLR-3 deficiency resulted in enhanced WNV replication in neurons *in vitro*; *in vivo*, TLR-3$^{-/-}$ mice have greater WNV replication in the CNS and increased lethality (7). While TLR3 is dispensable for protection from HSV-1 infection in mice (35), the loss of its downstream signaling molecule, IRF-3, rendered mice susceptible to CNS infection in the current study. Together, these data demonstrate the importance of early recognition sensors and IRF-3 dependent pathways in the control of viral infection of the CNS. By initiating a timely type I IFN response, IRF-3 dependent pathways limit initial CNS viral replication; in their absence, viruses like HSV-1 can establish a more robust CNS infection that can lead to death. Therefore, studies examining modulation and disruption of the IRF-3 dependent pathways must continue. Recent work utilizing high throughput screening of a pharmacologically active compound library has revealed several antipsychotic drugs to be direct inhibitors of innate signaling pathways (36). Patients receiving these drugs might be more susceptible to HSE, similar to what has been reported in genetic studies for TLR-3 deficiency.

The results from HSV-1 and WNV infection also suggest a neuroprotective role for IRF-3 either through induction of type I IFN or an IRF-3 dependent cascade. A recent study demonstrated that viral infection is not a requirement for protection provided by IRF-3 in the brain. Preconditioning of the mouse brain with lipopolysaccharide (LPS) protected the mouse from ischemic injury following cerebral artery occlusion; this protection was found to be dependent on type I IFN production via IRF-3 dependent pathways (22). Together, the data suggest that IRF-3 may be a suitable therapeutic target

for IFN induction in the brain. Direct treatment within type I IFN has been utilized in a variety of human illnesses including hepatitis virus infection, multiple sclerosis and gliomas (4, 11, 32). However, several major problems have been identified in utilizing IFN treatment, most notably a short half life *in vivo* and inaccessibility to the CNS (27). In addition, IFN treatment has been associated with numerous clinical side effects including depression and brain toxicity (27) . By targeting IRF-3 in the brain, treatment may lead to physiological appropriate amounts of IFN being produced in the CNS by local cells; the result may be increased half-life for IFN in targeted tissues, lowered toxicity, and decreased side effects. Targeting IRF-3 might also be used as a prophylactic treatment to prevent viral encephalitis if a person is known to be infected with or exposed to HSV-1 or another neurotropic virus. Whereas a delay in type I IFN induction result in augmented viral replication the CNS, early induction and priming via IRF-3 might limit infection. A recent study has identified a chemotherapeutic agent, 5,6-dimethyl-xanthenone-4-acetic acid (DMXAA), as a potent and specific activator of IRF-3 (28). Through a yet undetermined mechanism, DMXAA activates IRF-3 pathways in an IPS-1 and MYD88 independent manner and, unlike LPS, fails to induce TNFa expression. Together, DMXAA and other drugs that target IRF-3 may prove to be an effective therapeutic in treatment of viral infection of the CNS.

In addition to the necessity of IRF-3 in the CNS, these *in vivo* experiments illustrate the importance of inspecting several routes of infection. The initial studies examined HSV-1 infection following intravenous inoculation (14). In such infections, the virus would be expected to be shuttled to the liver or spleen, each sites with considerable immune cell populations. The results demonstrated that in such an

experiment, IRF-3 had minimal impact whereas IRF-7 deficient mice had significant lethality *in vivo* (14). However, this method bypasses the tissues associated with physiological HSV-1 infections: the epithelia and neuronal cell. In this study, examination of the ocular route of infection permitted evaluation of the full HSV-1 lifecycle. While replication was unaffected in the epithelial tissues and the sensory ganglia, the brain demonstrated increased susceptibility. Intravenous infection likely provides minimal exposure to brain tissue and thus underestimated the role of IRF-3 in limiting HSV-1 infection. For the same reason, in addition to peripheral infection, intracranial injection has been utilized to explore HSV-1 infection of the CNS. Numerous factors influence the ability of the virus to enter the CNS following peripheral infection; therefore, to adequately evaluate neurovirulence versus neuroinvasiveness, direct injection of the virus into the brain is required. In each case, the differing routes of infection answered different *in vivo* questions. Intravenous infection examines the immune response to a systemic infection. Ocular challenge examines physiologically relevant portions of HSV-1 infection cycle and intracranial injection evaluates neurovirulence and fitness in the brain. Reliance on a single route or model of infection, however, fails to adequately evaluate the immune components being studied.

Future Directions

An immediate area of interest involves further characterization of HSV-1 infection in IRF-$3^{-/-}$ brains. The presented data demonstrates increased viral replication due to a deficit in type I IFN production in IRF-$3^{-/-}$ mice. In combination with inflammation, increased viral replication leads to increased lethality in IRF-$3^{-/-}$ mice as compared to controls following HSV-1 challenge. Interestingly, while increased antigen

production and distribution was observed in IRF-3$^{-/-}$ brain sections, the staining pattern was also distinct compared to WT mice. As mentioned previously, HSV-1 staining in IRF-3$^{-/-}$ brain sections had generalized antigen positive lesions contrasting focal staining found in WT brains. One possible explanation suggests that the absence of IRF-3 simply results in increased antigen production in the infected brain regions. An alternative explanation suggests a possible shift in tropism with HSV-1 infection of support cells in addition to neurons. While some HSV-1 infection of support cells like astrocytes and glial cell have been reported, neurons are typically the target of HSV-1 infection (20-21, 34). Utilizing dual fluorescence staining, changes in viral tropism in IRF-3$^{-/-}$ can be determined with specific markers for neurons, astrocytes, microglia, and other cell types found within the brain. Further examination these cell types *in vitro* may also reveal a shift in susceptibility in the absence of IRF-3, similar to BMDC and BMM. Initial examination of cortical neurons demonstrated no change in HSV-1 replication in IRF-3$^{-/-}$ cultures as compared to controls (Fig 4.1A); however, primary IRF-3$^{-/-}$ astrocyte cultures demonstrated increased viral replication in initial experiments (4.1B).

A second area of interest involves the continued defect of IRF-3$^{-/-}$ BMDCs in controlling HSV-1 replication following IFN pretreatment. One possibility is that defective autocrine/paracrine IFN amplification or the loss of specific ISGs result in the failure to restore complete control of viral replication in IRF-3$^{-/-}$ BMDCs. An alternate hypothesis is that the virus recognition machinery may be required to augment the ongoing immune response. In the absence of type I IFN signaling, BMDCs fail to mature following antigenic stimulations (18). In these studies, IRF-3$^{-/-}$ BMDCs have delayed production in type I IFN and therefore, a likely delay in maturation. With few

exceptions, examination of cytokine profiles revealed a global delay in cytokine production from IRF-3$^{-/-}$ BMDCs compared to WT following HSV-1 challenge (Fig 4.2). In addition, upregulation of CD86 also lagged behind WT BMDCs following HSV-1 infection (Fig 4.3). Interestingly, treatment with IFNβ resulted in CD86 upregulation in WT BMDCs but not in IRF-3$^{-/-}$ BMDCs. This data suggest a possible explanation for the continued defect in IRF-3$^{-/-}$ BMDCs following IFN pretreatment. The lack of IRF-3 potentially hinders BMDC activation either through reduced IFN amplification, loss of an IRF-3 dependent gene product, or absence of a signaling cascade that indicates infection. In contrast, treatment with poly IC induced upregulation of CD86 in both WT and IRF-3$^{-/-}$ BMDC and provides evidence that pattern recognition contributes to BMDC maturation.

Having determined an impact for IRF-3 dependent pathways on HSV-1 infection both *in vitro* and *in vivo*, the pathways leading to HSV-1 recognition and subsequent IRF-3 activation remained to be determined. Previous reports had identified numerous sensors and adaptors involved in the recognition of HSV-1, yet none had been shown to have an impact on viral replication. Using the BMDC cultures, a variety of immune component knockouts were screened for their control of HSV-1 replication; IRF-3$^{-/-}$ BMDCs served as a positive control for enhanced viral replication. The results demonstrated that none of the tested immune knockouts recapitulated the *in vitro* phenotype seen in IRF-3$^{-/-}$ BMDCs (Table 4.1). Known sensors and adaptors from the toll-like receptor and RIG-I like receptor pathways had no significant increase in viral replication when compared to control cells. These results suggest that either the pathway involved in recognition of HSV-1 has yet to be identified or has yet to be tested. Another

possibility is that multiple sensors and adaptors are involved in HSV-1 recognition and compensate for the absence of another pathway.

Concluding Remarks

The studies herein demonstrate a role for IRF-3 in the control of HSV-1 infection both *in vitro* and *in vivo.* The loss of IRF-3 directly impacts the kinetics and production of type I IFN and thus renders IRF-$3^{-/-}$ cells and mice more susceptible to HSV-1 infection. The study illustrates the importance of IRF-3 mediated recognition pathways in the control of viral infection. We hope that these experiments provide a foundation for further examination of the early recognition pathways and provide novel insight into the interaction between HSV-1 and immune host defenses.

Figure 4.1. Preliminary growth curves from primary IRF-3$^{-/-}$ brain cell cultures. Primary (A) cortical neurons and (B) astrocytes were infected with HSV-1 at MOI 0.01. At indicated times post infection, samples were collected and assayed for viral replication via plaque assay on Vero cells. Results shown are representative of two independent experiments.

Figure 4.2. IRF-3$^{-/-}$ BMDCs have a global delay in cytokine production as compared to WT BMDCs. WT and IRF-3-/- BMDCs were infected at MOI 5 and media harvested at 3, 6, 9, and 12 hours post infection. Bead based cytokine assays (Bioplex, BioRad) were performed and cytokine titers are expressed as pg/mL. Figures shown represent average of three independent experiments.

Figure 4.3. Maturation was delayed in IRF-3$^{-/-}$ BMDCs as measured by CD 86 upregulation. BMDCs were mock treated, infected with HSV-1 MOI 5, or treated with poly IC or 100 units IFNβ as specified. Twelve hours post infection, cell were harvested and assayed by FACs. Cells were gated on CD11b positive, PI negative populations and assayed for upregulation of CD86, a measurement of BMDC activation.

Table 4.1. Summary of immune deficient BMDCs challenged with HSV-1 Infection. BMDCs were infected at MOIs of 1 and 0.01 and viral titers assayed at 6, 24, and 48 hours post infection. Each immune deficient BMDC was scored for increase in viral replication compared to control cells at either MOI or any time point.

Figure 4.1. Preliminary growth curves from primary IRF-3$^{-/-}$ brain cell cultures.

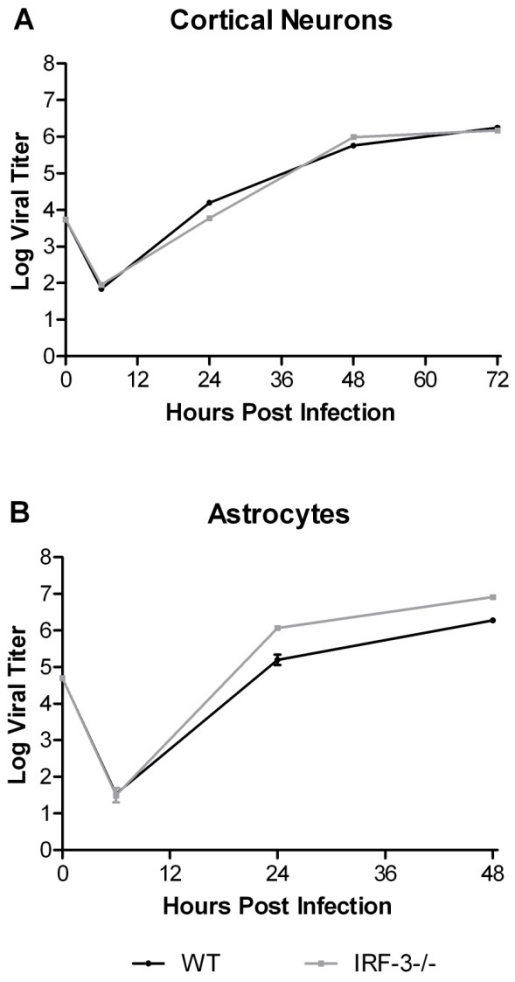

Figure 4.2. IRF-3$^{-/-}$ BMDCs have a global delay in cytokine production as compared to WT BMDCs.

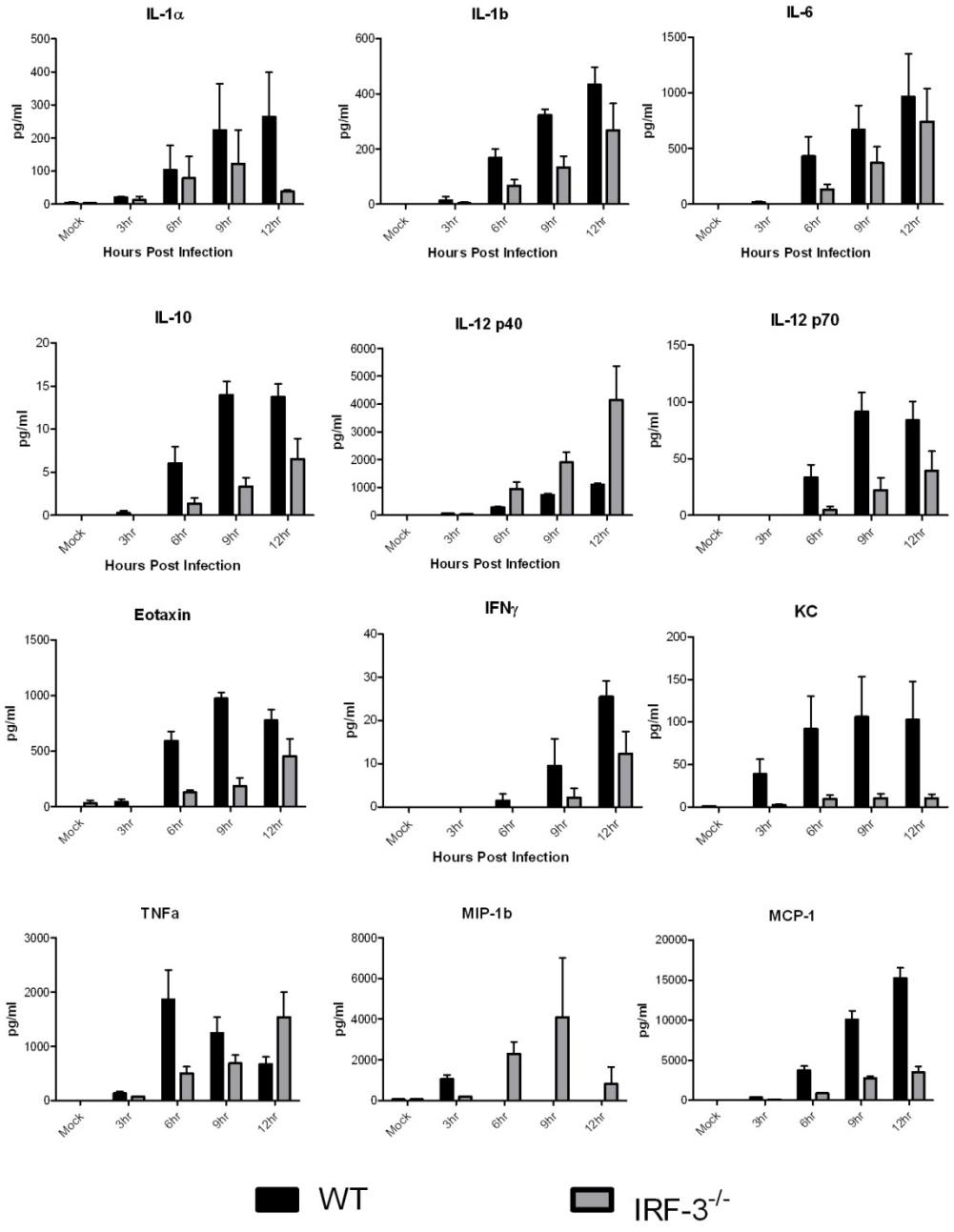

Figure 4.3. Maturation was delayed in IRF-3$^{-/-}$ BMDCs as measured by CD 86 upregulation.

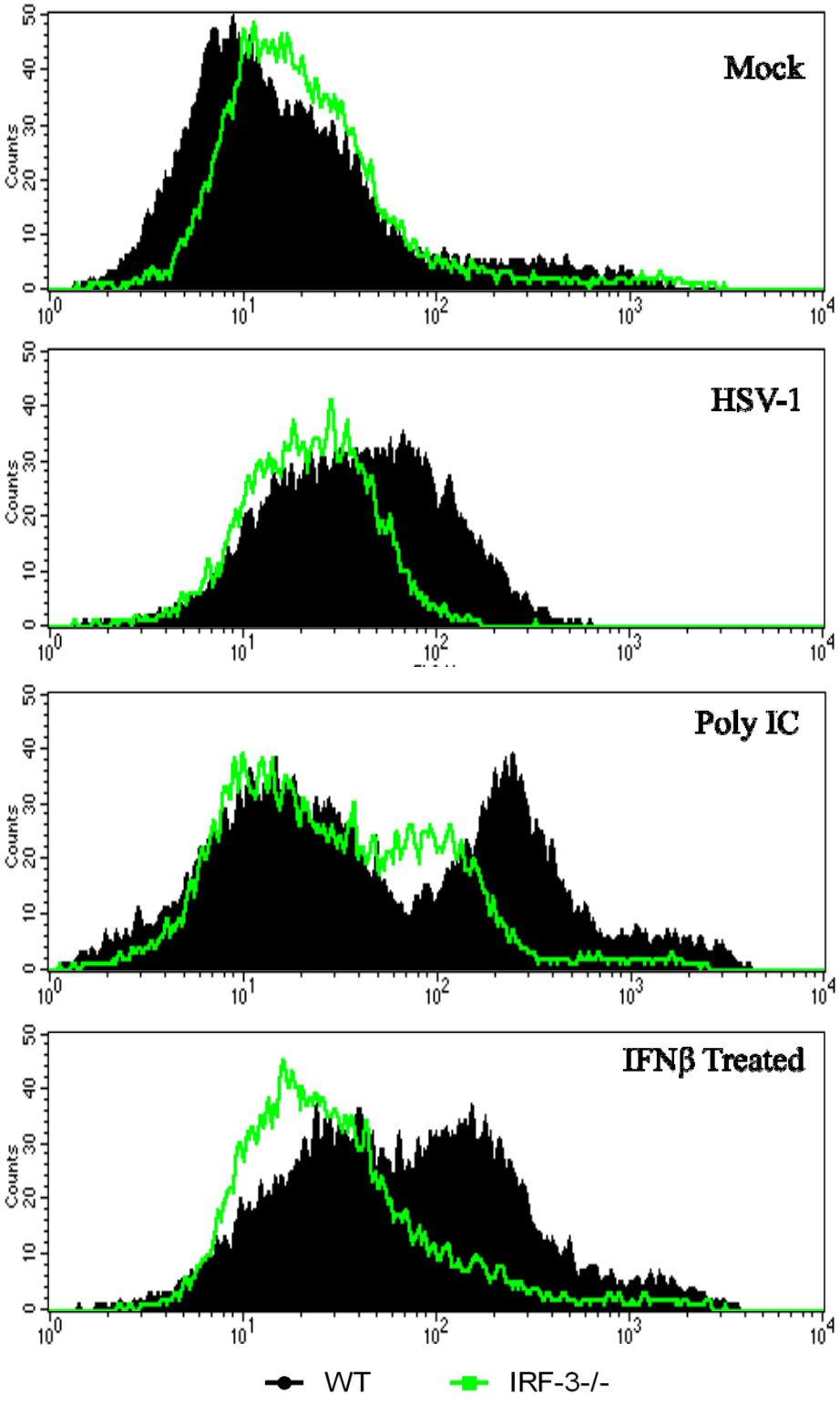

Table 4.1. Summary of immune deficient BMDCs challenged with HSV-1 Infection.

Knockout BMDC	**HSV-1 Infection** (relative to WT)
IRF-3-/-	Increased viral replication
IFNαβγR-/-	Increased viral replication
STAT-1-/-	Increased viral replication
IRF-7-/-	Equivalent to WT
MYD88-/-	Equivalent to WT
MYD88-/-, Trif-/-	Equivalent to WT
IPS-1-/-	Equivalent to WT
TLR-3-/-	Equivalent to WT
TLR-9-/-	Equivalent to WT
TLR-7-/-	Not Completed
RIG-I-/-	Equivalent to WT
MDA-5-/-	Equivalent to WT
MDA-5-/-, TLR-3-/-	Equivalent to WT
DAI-/-	Not Completed
STING-/-	Not Completed

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