MECHANISMS OF TYPE-I IFN INHIBITION: EQUINE HERPESVIRUS-1 ESCAPE FROM THE ANTIVIRAL EFFECT OF TYPE-1 INTERFERON RESPONSE IN HOST CELL

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MECHANISMS OF TYPE-I IFN INHIBITION: EQUINE HERPESVIRUS-1 ESCAPE FROM THE ANTIVIRAL EFFECT OF TYPE-1 INTERFERON RESPONSE IN HOST CELL

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

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2019

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ABSTRACT OF DISSERTATION

MECHANISMS OF TYPE-I IFN INHIBITION: EQUINE HERPESVIRUS-1 ESCAPE FROM THE ANTIVIRAL EFFECT OF TYPE-1 INTERFERON RESPONSE IN HOST CELL

Equine herpesvirus-1 (EHV-1) is one of the most important and prevalent viral pathogens of horses causing a major threat to the equine industry throughout most of the world. EHV-1 primarily causes respiratory disease but viral spread to distant organs enables the development of more severe sequelae; abortion and neurologic disease. In order to produce disease, EHV-1 has to overcome the innate barrier of the type-I interferon (IFN) system in host cells. However, the underlying mechanisms employed by EHV-1 to circumvent the type-I IFN response in host cells are not well understood. In this project study, using molecular techniques, we explored how EHV-1 is able to escape the type-I IFN response in host cells during infection. We also investigated whether EHV-4, a closely related but less pathogenic virus, has similar effects on type-I IFN as a clue to understanding how widespread IFN suppressive function is found among equine alphaherpesviruses.

Our data showed that inhibition of the type-I IFN response in host cells is not a function of neuropathogenicity of EHV-1 strains. However, a reduced type-I IFN response correlated with pathogenicity as EHV-4, unlike EHV-1, was unable to down-regulate the type-I IFN response in equine endothelial cells (EECs). Investigation of the mechanisms employed by EHV-1 to suppress type-I IFN revealed that the virus sequentially prevented outside-in signaling events that lead to type-I IFN production. Specifically, EHV-1 blocked the expression of Toll-like receptors (TLR) 3 and TLR4 at 6 hours post-infection (hpi) and 12 hpi. EHV-1 also prevented the transcription of IRF7 and IRF9 at different time-points during infection. The virus also perturbed the JAK-STAT signaling pathway by negatively regulating the cellular levels of TYK2 and phosphorylation-mediated activation of STAT2 molecules. Immunofluorescence data revealed that during infection, EHV-1 was able to sequester STAT2 molecules from nuclear translocation. This may be a limiting step preventing the formation of interferon-
stimulated gene factor 3 (ISGF3) whose nuclear translocation is required to transactivate interferon-stimulated genes (ISGs) including IRF7.

Further investigation showed that unlike EHV-1, EHV-4 only interfered with phosphorylation-mediated activated STAT1 and STAT2 molecules at 3 and 6 hpi. EHV-4 was unable to block TLR3/4 and IRF7/9 mRNA expression at any time-point. Intriguingly, while viral late gene of EHV-1 mediates inhibition of STAT phosphorylation, our data showed that for EHV-4, a virus late gene did not mediate the inhibition of STAT phosphorylation. The findings from this study help illuminate how EHV-1 strategically interferes with limiting steps required for type-I IFN response in host cells to promote pathology. Our data also strengthen the hypothesis that the ability to shut off host factors required for type-I IFN production might be directly related to the degree of pathogenicity of the EHV subtypes.

KEYWORDS: EHV-1, STAT2, innate immunity, type-I interferon, horse, EHV-4

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September 13, 2019
Date
DEDICATION

I dedicate this work to God almighty for seeing me through the many difficulties that life poses to an adventurist.
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CHAPTER 1: LITERATURE REVIEW

1.1 Research overview

Equine herpesvirus 1 (EHV-1) was first described by Dimock and Edwards in the early 1930s (1). Since then, EHV-1 infection and immunity have gained prominence in the literature (2-6). However, over 80 years after its first description and despite many vaccine trials, the virus remains one of the most important and prevalent viral pathogens of horses and a major threat to the equine industry (7). Almost all domesticated horses are repeatedly exposed to this virus and, as a result, may experience significant morbidity and even mortality (8). Depending on host or viral factors, exposure to EHV-1 can result in respiratory disease, sporadic abortion during the third trimester of gestation, neonatal deaths, chorioretinitis, and neurologic disease (9-11). Additionally, the disease is highly contagious among horses. One of the most devastating manifestations of EHV-1 infection is the neurologic form - equine herpesvirus myeloencephalopathy (EHM). Although EHM is not new, more outbreaks are being recognized with more horses seeming to be affected in each outbreak, causing concern among many horse owners.

This viral infection is unique in its ability to escape the host immunity and progress to latency after primary infection within the first weeks or months of life (12), leading to a carrier state in infected horses. Most horses acquire the infection at a young age and remain latent carriers for the rest of their lifetimes (5, 10). Latent infection occurs in the trigeminal ganglion and lymphoreticular system (13). The latent virus can recrudesce into full-blown productive infection,
typically when the animals are under stress, with the potential for subsequent shedding and infection of other susceptible horses, or abortion in the latently infected horse itself (14). Additionally, the disease is highly contagious among horses. One of the most devastating manifestations of EHV-1 infection is the neurologic form - equine herpesvirus myeloencephalopathy (EHM). Although EHM is not new, more outbreaks are being recognized with more horses seeming to be affected in each outbreak, causing concern among many horse owners.

Interferons (IFN) are a group of biological regulatory proteins, or cytokines, which act as an early line of defense against viral infections (15). Three classes of IFN, Type-I, II and III, have been characterized (15). In general, type-I IFNs which include multiple IFN-β and IFN-α species, along with IFN-ε, -δ, -ω, and -κ (16) are produced by a wide variety of cells including fibroblasts, epithelial cells, monocytes, macrophages, leukocytes and dendritic cells after viral infection. Type-II interferon on the other hand includes IFN-γ and it is produced by activated NK, NKT cells and macrophages after viral invasion of the host cell (17, 18). In 2003, a third type of interferon response (IFN-λ) against viral infection was identified in the host cell (19, 20) and it utilizes a heterodimeric receptor that is different from the receptors of either type-I or type-II interferons. Of the three types of IFN response, the type-I IFN system is potentially the most important host defense against a systemic viral infection. Upon viral infection of a susceptible cell, type-I IFN (IFN-β) is induced in the infected cell which then binds to cognate interferon α receptors (IFNAR1 and IFNAR2) on both the infected and
uninfected cells (21). This triggers a signaling cascade that subsequently leads to the activation of the transcription factors which are responsible for the production of a diverse variety of antiviral genes called interferon stimulated genes (ISGs) (22). The release of ISGs helps to create a global antiviral environment in the infected host animal to curtail viral replication and viral cell-to-cell spread. The importance of the type-I IFN response is not only explained by its function in the immediate early phase of the nonspecific cellular response but also in serving as a link between the innate and the adaptive arms of the host immunity (23-25).

For instance, IFN-α/β induces natural killer cell cytotoxicity and up-regulates expression of major histocompatibility complex class I on most cells and costimulatory molecules on antigen-presenting cells (26, 27). Furthermore, type-I IFNs enhance cross-presentation of exogenous antigen in major histocompatibility complex class I and promote T-cell expansion (28-30).

Over the years, many research studies have focused on the role of the adaptive immune response in curtailing the replication cycle of EHV-1, but little is known about the ways by which EHV-1 can manipulate and overwhelm the host type-I IFN response prior to disease progression. In addition, the biological functions of most of the EHV-1 viral proteins in the infected host cell remain poorly understood. Recently, Sarkar et al reported that EHV-1 gene expression was necessary to interfere with STAT-1 phosphorylation and nuclear translocation (31). The nature or the identity of the viral genes involved in the prevention of key steps of type-I IFN induction is yet to be determined. In order to come up with the development of viable interventions that will limit the scourge of
this infectious disease, detailed knowledge of how the virus outsmarts the host type-I IFN system is highly fundamental. The central focus of this research hinged on identifying the key pathways of the type-I IFN response that are downregulated by EHV-1 in the course of an infection using an equine endothelial cell model. The next section describes what is currently known about equine herpesviruses with a central focus on EHV-1, its disease manifestations, and host immunity against EHV-1 with particular emphasis on type-I IFN system.

1.2 Historical perspectives of the herpesviruses

Within the virus kingdom, Herpesvirales is a huge order that includes numerous viruses infecting humans and almost all animal species, including insects, fish, mollusks, reptiles, birds, and mammals (32). The first written documentation of the word herpes was by Hippocrates (460 BC-377 BC) (33-35) and possibly most of the disease conditions now designated as being herpes would not have been called by that name before Hippocrates’ documentation. In ancient time, the word ‘herpes’ became commonly used to describe a diverse range of spreading cutaneous lesions of varied etiology (36). It is known that the word ‘herpes’ has been used in human medicine for at least 2,600 years due to early descriptions of the disease-causing eczema and cancer of the skin (33). With time, the term was restricted to skin eruptions with a vesicular component, and by the latter part of the nineteenth century, a distinction was made between lesions caused by poxviruses and herpesviruses based on clinical and epidemiologic features (37). The term ‘herpes’ comes from the Greek word herpein which implies to creep or crawl beneath the skin and therefore
herpesvirus derived its name from this word as a result of the characteristic
nature of the disease which involves spreading of cutaneous lesions. Different
scientists have provided a fascinating history of the usage of the terms (33, 35)
and selected usage was illustrated by Onions (38), but of the many clinical
conditions, only a few, i.e., herpes simplex, herpes labialis, herpes genitalis, and
herpes zoster are still designated by this term (39).

The earliest herpesviruses were named after the clinical conditions or the
diseases they cause. Herpes simplex virus, herpes zoster virus, Aujesky’s
disease virus (pseudorabies), and Marek’s disease virus are examples of such
designations. The modern notion of herpes probably began with the definitions of
Willan and Bateman (40). The term was restricted to conditions characterized by
the appearance of localized groups of vesicles, a short self-limiting course, and
the absence of more than mild constitutional symptoms (41). The species of
herpes included zoster, labial herpes, and genital herpes; also included were
ringworm (H. circinatus) and erythema multiforme (H. iris) (41). These later
conditions were still classified as species of herpes until as late as 1880 and
appear at least as late as 1938 in the “American Illustrated Medical Dictionary”
(42). Vidal was able to demonstrate the infectious nature of herpes simplex in
humans (43). Similarly, herpes zoster was shown to be infectious by Von Bokay
(44), who also suggested as early as 1888 that zoster and chickenpox were
related. Even though the genomes of sequenced mammalian herpesviruses
differ widely, there is a subset of around 40 genes that are conserved by criteria
of encoded amino acid sequences and local preservation of gene layout (45-48).
This suggests that these viruses evolved from common ancestors but have diverged to a greater extent over time. While herpesviruses have been considered to be evolving along with their hosts over about 400 million years, the subgroup alphaherpesviruses, to which EHV-1 belongs, diverged only 180-210 million years ago (49, 50). Thus, equine herpesvirus 1 likely has been plaguing wild or domestic equid populations much earlier than the first written documentation of the disease which occurred in the early 1930s.

1.3 EHV-1 infection of horses: How it all began

The discovery of the first virus, Tobacco Mosaic Virus, is attributed to the work of Dmitry Iosifovich Ivanovsky, a Russian-Ukrainian scientist considered the father of Virology (51). However, after that discovery, fifty years passed before William Wallace Dimock and Philip Edwards in 1932 first showed that a different kind of microorganism, other than bacteria, was causing contagious epizootic abortion in mares (52). In the same year, the disease was reproduced by experimental inoculation of materials from aborted fetuses into mares. Their research indicated that a filterable viral agent was causing abortions in pregnant mares, and they coined the term “viral abortion” to refer to the syndrome (1). They went further to describe the gross pathological changes of aborted fetuses, including intranuclear inclusion bodies in the fetal pneumocytes and hepatocytes, and defined the clinical observations of ‘equine viral abortions’ (53). Later, the ‘equine abortion virus’ (EAV) was cultivated in laboratory animals and in tissue culture (54-56), and more extensive pathological findings were published (57).
Around the same period, Manninger and Csontos in Hungary also documented the same symptoms of viral abortions as in Kentucky, along with additional syndromes of respiratory diseases including mild fever (58). They observed the development of symptoms resembling that of mild influenza when bacteriological sterile filtrate from the aborted fetuses with lesions of viral abortion was inoculated into pregnant mares (58). Salyi also demonstrated that the observed gross and microscopic lesions in fetal abortion material were identical with those reported in Kentucky (59). Kress further suggested that the abortion virus was pneumotropic because of occurrence of bronchopneumonia in horses that had been in contact with aborted mares and fetuses (60). This led Manninger to conclude that viral abortion was due to infection of pregnant mares with equine influenza virus (61).

The respiratory infection associated with EAV was first studied experimentally by Doll et al. (62), and the symptomatology produced in inoculated young horses was again similar to that described as equine influenza whose cause had not yet been identified. The evidence from their study showed that EAV was the etiological agent of an epizootic respiratory disease of young horses (62). It remained for Doll and associates to prove that several putative influenza virus isolates were identical with EAV (63-65). In another study, Bryans and co-workers suggested that the causative agent previously known as EAV should be regarded as a respiratory virus because the principal histological lesions in young horses and aborted foals occur in the respiratory tract (66). Accordingly, the authors designated the disease caused by the virus as viral
pneumonitis and the agent as an equine viral pneumonitis virus. In 1963, the virus was shown by electron microscopy to be a member of the herpes group (67).

1.4 Classification of herpesviruses

In the course of their evolution, herpesviruses have undergone considerable diversification with respect to virion morphology, biological properties, and virion antigenic properties (39). The members of the Herpesviridae family are divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae (68) based on their morphology and biological properties.

Alphaherpesviruses are found in a wide range of host species. They undergo efficient and relatively short replicative cycle, and they establish latency in the sensory neurons or lymphocytes of their hosts (69). They spread well from cell to cell but they also are easily released from infected cells, in which they multiply causing cytopathic effect and formation of eosinophilic intranuclear inclusion bodies (70). In vitro, they can often infect cells originating from varying species of animals. Although in vivo the alphaherpesviruses can infect various host species, there is always a specie to which each virus has been adapted (70). In such a host, they have the propensity to undergo latency, during which viral pathogenicity is absent. Within the host, the alphaherpesviruses are believed to spread best along the nerves, where intra-axonal transmission predominates (70). Members of Alphaherpesvirinae subfamily include four
different genera; Simplexvirus, Varicellovirus, Mardivirus, and Iltovirus (71). EHV-1 is classified as a member of the Varicellovirus genus.

Unlike alphaherpesviruses, betaherpesviruses have a restricted host range and a long reproduction cycle (72). In vitro, members of the Betaherpesvirinae only replicate in cells derived from their specific host, further underscoring their narrow host range (70). Their replication cycle is slow (lasts several days) and their release from infected cells is inefficient (70). Betaherpesvirus infection slowly progresses in tissue culture and the infected cells become larger rather than lyse and contain intranuclear inclusion bodies (70, 73). Latent infection is established predominantly in monocytes or macrophages (72). Because they do not show the preferential neural spread, they usually persist in leukocytes, in cells of the reticuloendothelial system and also in epithelial cells of renal tubuli and salivary gland ducts (70). The viruses in this subfamily are subdivided into four genera namely Cytomegalovirus, Muromegalovirus, Roseolovirus, and Proboscivirus (74).

The members of the subfamily Gammaherpesvirinae are slow replicating viruses with lymphotropic properties and limited host ranges (70). In contrast to both alpha- and betaherpesviruses, gammaherpesviruses seem to initially favor the establishment of latency in either T or B cells, while only a subset of cells supports lytic replication (75). There are more homolog genes conserved within members of the subfamily Gammaherpesvirinae than members of the other two subfamilies (72). In addition to the genes conserved between herpesviruses, each gammaherpesvirus also contains a set of unique genes which are usually
present at the terminal regions of the genome and which are important for viral pathogenesis (72). This subfamily consists of four genera: *Lymphocryptovirus*, *Rhadinovirus*, *Macavirus*, and *Percavirus* (74).

### 1.5 Equine herpesviruses

To date, all the nine equid herpesviruses isolated belong to either the *alphaherpesvirinae* or *gammaherpesvirinae* subfamilies (Table 1.1). The members of the subfamily of alphaherpesviruses include EHV-1, EHV-3, EHV-4, EHV-6, EHV-8, and EHV-9 (76). The members of the gammaherpesviruses include EHV-2, EHV-5, and EHV-7. Only five of the nine herpesviruses (viz. EHV-1, 2, 3, 4 and 5) can produce disease in horses (77). EHV-6 to 8 infect donkeys and are also called asinine herpesvirus (AHV, AHV-1 to 3), while EHV-9 or gazelle herpesvirus (GHV) infects Thomson's gazelles (78-80).

### 1.6 Genomic structure and gene functions of EHV-1

The complete genome sequence of EHV-1 has been published (81, 82) making information regarding the genomic organization of EHV-1 available. EHV-1 has a linear dsDNA molecule of about 150.2 kbp in size with base compositions of about 56.7% G+C content (73). The genome is composed of a long unique region (UL, 112,870 bp) flanked by a small inverted repeat sequence (TRU/IRL, 32 bp) and a short unique region (US, 11,861 bp) that is flanked by a large inverted repeat (TRS/IRS, 12,714 bp) (73). The genome contains 80 open reading frames (ORFs), which encode 76 unique genes, with four duplicated ORFs present in the terminal repeat sequence (TRS) (82, 83). The four duplicated ORFs in the EHV-1 genome are ORF 64, 65, 66, and 67 which are
present in the sequences flanking the unique short segment (77). The inverted repeats allow the short components to give rise to virion populations which exist in two orientations, resulting in the formation of two isomeric DNA molecules (84-88). The gene layout of EHV-1 reveals tightly arranged ORFs with little intervening sequence, the absence of extensive ORF overlap, and few instances of exon splicing (77). Generally, this gene arrangement of EHV-1 is similar to other sequenced herpesviruses with the only difference being that EHV-1 encodes five genes ORF 1, 2, 67, 71, and 75 which have no structural homolog when compared to all other herpesviruses sequenced to date (77). The functions of some of these genes remain unknown but have been predicted to exert major influence in the unique biology of EHV-1 enabling them to adapt to the horse as their natural host (77). The genomic details of EHV-1 ORFs including the functions of individual genes are listed in Table 1.2.

1.7 Biological functions of EHV-1 proteins

The structural architecture of a purified EHV-1 particle (Fig. 1.1) is made up of about 30 discrete kinds of polypeptides (89-93). It consists of a genomic core made up of a linear double-stranded DNA neatly packed within an icosahedral capsid of T=16 with an approximate diameter of 100 to 110 nm (72). The nucleocapsid, which houses the viral genome, is in itself made up of six proteins encoded by ORFs 22, 25, 35, 42, 43, and 56 (77, 90). All herpesviruses have a similar capsid structure composed of 162 capsomers (12 pentons and 150 hexons) (94). The nucleocapsid contains a ring structure made up of 12 portal proteins which enables viral DNA to enter into the capsid (95, 96).
Although their names vary between herpesvirus families, the structure and arrangement of capsid proteins are conserved across all herpesviruses (97, 98).

The amorphous tegument layer, which corresponds to the area between the nucleocapsid and the envelope, comprises about twelve different proteins encoded by ORFs 11, 12, 13, 14, 15, 23, 24, 40, 46, 49, 51, and 76 (77). These tegument proteins and enzymes are critically involved in very early events during infection which are required for initiating viral replication (94, 99, 100). The large tegument protein, UL36, interacts with the pentons (VP5) of the capsid and this interaction gives the innermost part of the tegument an icosahedral symmetry (101-103). The outermost part of the tegument interacts with the virus envelope membrane and may sometimes come in contact with the transmembrane domains of envelope glycoproteins.

The nucleocapsid and the tegument are surrounded by an envelope from patches of the altered host-derived cell membrane (104). Embedded in the EHV-1 envelope are about eleven glycoproteins which are functional homologs of those found in HSV-1. The eleven glycoproteins of EHV-1 (i.e. gB-gp14, gC-gp13, gD-gp18, gE, gG, gH, gI, gK, gL, gM and gN) are conserved across all alaphaherpesviruses and therefore named according to the nomenclature established for HSV-1 (94). As with other herpesviruses, the envelope glycoproteins of EHV-1 are critical determinants of virus entry into a susceptible host cell, host range, virus cell-to-cell spread, pathogenicity, and immunologic responses to infection. EHV-1 encodes an additional gp2, which has homologs present only in EHV-4 and AHV-3 (94). The inclusion of tegument and viral
envelope enables the virion size to markedly increase from 120 nm to approximately 300 nm (73).

1.8 Epidemiology and transmission of EHV-1

It is estimated that between 80 to 90% of horses have been exposed to either EHV-1 or its close relative EHV-4 by the time they are 2 years of age (7). The great level of antigenic similarity between EHV-1 and EHV-4 often complicates seroepidemiological data as a result of lack of type-specific antibodies and extensive antigenic cross-reactivity that exists in natural infection (105). In the early 1990s, evidence became available that the envelope glycoprotein, gG, of EHV-4 elicits a type-specific antibody response, which for the first time provided the opportunity to differentiate between antibodies present in polyclonal sera from mixed cases of infection involving both EHV-1 and EHV-4 (106). The epitopes present in the carboxyl domain, a variable region of the gG’s of both viruses, were identified as useful tools for differentiating between EHV-1 and EHV-4 based on distinct type-specific humoral responses that they elicit in their natural hosts (106, 107). The annual incidence of EHV-1 is not well defined, as a result of mixed infection with EHV-4 and the ability of both viruses to undergo latency. Latency is an important pathological strategy employed by alphaherpesviruses for continuous survival and spread within the natural host population (108). The virus can be reactivated at any time during the life of the infected host to promote a clinical course of the disease and virus shedding.

EHV-1 is a highly contagious viral infection and is easily spread by direct contact, fomites, and aerosols after shedding from the nasal cavity or by the high
virus loads present in aborted materials including placenta (109). Transmission of the virus to susceptible horses is facilitated by contact with an acutely infected horse or a reactivated virus-shedding horse or from contact with aborted fetuses or placenta which is rich in infectious virus particles (77). Extensive work investigating the transmission cycle of EHV-1 has identified mare and foal populations as important reservoirs enabling virus transmission before and after weanling, with foals becoming infected as early as 30 days of age (110). In another study, EHV-1 shedding was reported in 22 day-old foals even after a widespread vaccination of mares (12). Evidence suggests that infected mares, especially the latently infected ones, serve as a continuous source of EHV-1 exposure to foals by horizontal transmission when contact is established between foals and the nursing dam. Broodmares may undergo recrudescence of latent viral infection as a result of stress resulting from pregnancy/parturition which may expose young foals to EHV-1 infections from mares that are actively shedding the virus (94). Overall, available data suggest a cyclic but mostly quiet epidemiologic pattern of EHV-1 infection with the dams serving as a continuous source of infectious virus particles to their foals between breeding seasons.

1.9 Cell infection and virus replication

The viral life cycle can be divided into the following major steps: entry into a permissive host cell, uncoating of viral nucleocapsid, expression of viral genes, viral DNA replication, virion assembly, and egress of newly produced virion particles (Fig.1.2). In horses, EHV-1 can infect at least three distinct cell types in three different organ systems including epithelial cells, mononuclear cells in
lymphoid tissue and peripheral blood (PBMCs), and endothelial cells of inner organs (5). Cells are either infected by direct contact with an infectious EHV-1 particle or by cell-to-cell spread following contact with an infected cell in the same host (94). As with herpes simplex virus 1 (HSV1) and most other alphaherpesviruses including EHV-1, productive infection is initiated by a relatively unstable attachment to heparan sulfate moieties on cell surface proteoglycans that is mediated by gC and gB, followed by binding of gD to one of the specific receptors expressed on the cell surface (111-113). Receptor engagement leads to conformational changes enabling complex interactions between gB and gH/gL (113). However, for virus entry into host cells, EHV-1 also utilizes a unique receptor that is different from those described for other alphaherpesviruses (114). Equine Major Histocompatibility Complex I (MHC-I) molecules serve as entry receptors that bind gD of EHV-1 to facilitate virus entry into equine dermal (ED) cells as well as equine brain microvascular endothelial cells (115, 116).

EHV-1 can enter permissive cells either by fusion of its envelope with the host cell membrane or by cell-mediated endocytosis, producing a productive infection in both cases (117). Both entry pathways facilitate the release of viral nucleocapsid and tegument proteins into the infected host cell. As with other alphaherpesviruses, once the virus is released inside the host cell, the tegument proteins dissociate from the nucleocapsid and the capsid is transported along microtubules via dynein, a minus-end-director motor protein, to the nucleus of the cell (94). This mechanism of capsid transport is important especially in the
infection of cells such as neurons where the virus may have to travel a long distance away from the site of the infection to reach the nucleus (94, 118). Following the arrival of the capsid at the nucleus, the capsid directly binds to the nuclear pore complex (NPC) and extrudes its content into the nucleus leaving the capsid behind in the cytoplasm (119, 120). For HSV-1, the inner tegument protein UL36 (ICP1/2) which bears a nuclear localization signal (120), together with nucleoporins Nup358 and Nup214 which both bind either directly or indirectly to the capsid, facilitate this process (118). Seemingly, all these associations and interactions are necessary for the nuclear import of the viral DNA by importin β (121).

Transcription and replication of the viral genome, as well as the assembly of progeny virus, takes place within the nucleus (118). These events lead to the reorganization of the nucleus causing an increase in the size of the nucleus and disruption of the nucleolus and nuclear domain-10 (ND-10), and chromatin condensation and its subsequent destruction together with the destruction of the nuclear lamina in the late steps of infection (122-124). The coordinated transcription of EHV-1 genes is regulated by six regulatory proteins that are expressed as: one IE protein (IEP), four early proteins (EICP22P, EICP27P, EICP0P, and IR2P), and the late protein EHV-1 α-gene trans-inducing factor (ETIF or VP16) (125-130). This cascade starts with the tegument VP16 (HSV) homolog protein of EHV-1 acting as a transactivator of an IE (α) gene expression (131). During viral entry, VP16 is carried into the infected cell as a tegument protein and is required for efficient initiation of the lytic replicative cycle of the
The IE protein is encoded by ORF64 and synthesized by host cell RNA polymerase II (128, 133). The IE of EHV-1 is a 1487-amino acid (aa) polypeptide which lies within each of the two inverted repeats (134) and is essential for replication of the virus (135). During initial stages of infection, IE protein activates the expression of heterologous viral promoters, auto-regulates its own expression, and acts synergistically with the EICP22 and EICP27 to activate the expression of early (E or β) and late (L or γ) viral genes (136-139).

For the transcription of IE genes of HSV-1, the cellular transcription factor Oct-1 binds to a unique consensus sequence: 5′-GyATGnTAATGArATTCyTTGnGGG-3′ (where y is a pyrimidine base, r is a purine base, n is any base) that overlaps the transcription initiation site of the IE promoter (118, 140). VP16 protein then interacts with Oct-1 and together with HCFC1 protein form a complex which activates transcription of IE genes (118).

The next set of genes to be transcribed during EHV-1 infection are the E genes which encode additional viral regulatory proteins (EICP0, EICP22, and EICP27), as well as proteins required for replication of the viral genome (125, 129, 136, 141, 142). Early gene transcription occurs before the initiation of viral DNA synthesis and it is tightly regulated by IE protein (126). The IR2 gene is embedded within the IE gene and encodes an early protein that is a truncated form (aa 323 to 1,487) of the IE protein (143). Although IR2 protein can trans-repress the IE gene expression, it is unable to trans-activate E and L gene expression due to lack of a trans-activation domain of the IE protein encoded by amino acid residues 3 - 89 (144). The EICP0 gene encodes an early nuclear
phosphoprotein, about 419 bp, that can trans-activate all classes of EHV-1 promoters (125, 126). This EICP0 contains a conserved cysteine-rich zinc RING finger (C3HC4 type) near the N terminus that is essential for activation of the E (β) and L (γ1 and γ2) promoters (126). The EHV-1 IE and EICP0 proteins are potent trans-activators of EHV-1 promoters, but unlike in HSV-1, they do not function synergistically but exhibit an antagonistic relationship (126, 145). The E genes encode proteins involved in enhancing viral replication while the late genes encode the viral structural proteins (94). Following the model of HSV-1, it is known that once the E proteins are synthesized, viral DNA replication will be started. This involves the interplay of at least seven early proteins including the gene products of UL5, UL8, UL9, UL29, UL30, UL42 and UL52 (146-148). The initial step of HSV DNA replication involves the unwinding of the double-stranded helix by UL9 and/or ICP8 (UL29) proteins in the AT-rich regions of the oriL or oriS origins of replication (118). The latter has one copy in UL of the herpesviral genome and two copies in Us of the genome respectively (118). ICP8 binds ssDNA fragments, and UL9 binds specifically to oriS and unwinds it enabling the helicase-primase complex composed of UL5, UL8, and UL52 proteins to be loaded (118). Following unwinding of the dsDNA, a complex of viral DNA polymerase (UL30) and processivity factor UL42 synthesize the leading and the lagging strand of the DNA (118). This replication occurs in a rolling circle form termed ‘theta form of replication’, the mechanism of which has not yet been identified. This replication ensures the formation of long head-to-tail concatemers
of viral DNA which are then cleaved into individual units during packaging of viral DNA into capsids (149).

In addition to the seven viral proteins, some other cellular factors involved in the replication of the viral genome include DNA ligase, topoisomerase II, and various components of the DNA repair and homologous recombination systems (150). Another important factor for viral DNA replication is cellular chaperone protein Hsp90 which is essential for intranuclear localization of viral DNA polymerase (151). Some viral proteins such as thymidine kinase (UL23), ribonucleotide reductase (UL39, UL40), deoxyuridine triphosphatase (UL50), uracil N-glycosylase (UL2), and alkaline nuclease (UL12) participate in nucleotide metabolism, viral DNA synthesis, and DNA repair (118).

The production of late (γ) viral genes peaks only after viral DNA replication has commenced and requires ICP4, ICP27, and ICP8 for efficient levels of transcription (152). γ1 (leaky-late) genes such as major capsid protein ICP5, gB, gD, and ICP34.5, are expressed throughout infection, increasing in transcription only a few fold after DNA replication has occurred (152) while expression of γ2 (true-late) genes such as gC, UL41 (VHS), UL36, UL38, UL20, and gK, does not accumulate in appreciable amounts until after DNA replication (152). The increase in the expression levels of the late genes, especially those encoding for viral capsids, just after DNA replication has been initiated enables the assembly of progeny virion particles (118).

Figure 1.3 illustrates the pathway involved in herpesviral capsid formation. The assembly of the herpesviral nucleocapsid occurs in the nucleus first as a
DNA-free precursor capsid in the presence of scaffolding proteins just before viral DNA encapsidation (94, 153-155). The first step in the capsid formation involves the auto-catalytic assembly of a procapsid following the interaction of pUL19 and pUL6 with a scaffold composed of conserved pUL26 and pUL26.5 proteins (156). These proteins associate to form angular segments of the spherical procapsid with binding enhanced by scaffold-scaffold interactions and by the triplexes which link VP5 molecules (97). This is then followed by progressive enlargement of the angular segments, called partial procapsids, to form an enclosed spherical procapsid (102). Although the procapsid appears to be spherical rather than being polyhedral, it has the same diameter as the mature capsid (125nm), and the same T=16 icosahedral symmetry (97). In a similar fashion as the major capsid protein, the portal is thought to be integrated into the developing procapsid by forming a complex with scaffold proteins (157-160). Following the formation of the procapsid, the viral dsDNA genome is then packaged into the capsid mediated by a three subunit-complex of a virus-encoded protein called terminase (161). The transport of the virion genome into the capsid marks the exit of the scaffold proteins from the procapsid creating the polyhedral shape of the mature capsid.

An early step in the nuclear egress of herpesviruses is the budding process at the inner nuclear membrane where the capsid, surrounded by tegument proteins, acquires an envelope derived from the inner leaflet of the nuclear membrane (162). After the viral genome has been packaged and assembled, the nucleocapsid travels within the nucleus with the aid of actin.
filament (163) to establish contact with the inner nuclear membrane before primary envelopment (Fig. 1.4.). Intimate contact with the inner nuclear leaflet is attained once the nuclear lamina has softened and at least partially dissolved requiring two virally encoded proteins, pUL31 and pUL34 (164) that are structurally and functionally conserved across herpesviruses (156). Complex formation between these two proteins is a prerequisite for primary envelopment and the absence of either protein stalls the process of nuclear egress tremendously (165-169). The association of the pUL31-pUL34 complex with nuclear lamins A/C or B (164, 170) leads to the recruitment of cellular protein kinase C (PKC) which then phosphorylates intranuclear lamins A/C and/or B (171). This complex interaction results in the dissolution of the nuclear lamin network and the underlying chromatin layer (124, 164), enabling the nucleocapsid to make contact with the inner nuclear membrane (156). Although the nucleocapsid acquires its primary envelope through the process of budding from the inner nuclear membrane, there is a striking difference in morphology and protein content when compared to the mature virus (172, 173). While the primary envelope contains both pUL31 and pUL34 proteins (164, 166), the mature virus particle lacks these two proteins demonstrating the differences in composition between primary and matured virions (156). The underlying mechanism by which the enveloped nucleocapsid gain access into the cytoplasm is not well understood. However, it has been shown that access to the cytoplasm is by fusion of the enveloped nucleocapsid with the outer nuclear membrane (156) rather than exit through the nuclear pore. This eventually leads to loss of
envelope (de-envelopment) enabling the naked nucleocapsid to acquire tegument proteins once inside the cytoplasm (162). The phosphorylation of a component of primary-enveloped virions achieved by the kinase activity of pUS3, a component of these particles in itself, is required for a successful de-envelopment process (174-176).

Final tegumentation and secondary envelopment occur in the cytoplasmic compartments and require a highly coordinated network of protein-protein interactions (156). The herpesviral tegument proteins interact with the capsid on one side and with the cytoplasmic tails of the envelope glycoproteins on the other side enabling the structural integrity of the matured virus particle (162). Two subassemblies, the capsid and the future envelope, are distinct sites where final tegumentation takes place and they efficiently combine to produce the mature virion (156). The capsid proximal proteins consist of conserved pUL36 and pUL37 that contribute to the physical structure of the tegument, the conserved pUL25, and pUS3 which remains closely associated with the capsid (156). Except for pUS3, the other identified components of the inner tegument are conserved in the Herpesviridae. Both pUL36 and pUL37 remain closely associated with the incoming capsid until they dock at the nuclear pore (177, 178) and also serve as a vehicle for intracytoplasmic transport of the capsid during entry and exit of the cytoplasm (179, 180). Besides these conserved components, additional non-conserved proteins may be associated with inner tegument (156). Strikingly, the level of the inner tegument proteins pUL36 and pUL37 in the virion is strictly regulated unlike those of the outer tegument which
vary extensively (181). The addition of the outer tegument is accompanied by virion secondary envelopment. This process of final envelopment occurs within the trans-Golgi network, where glycoproteins together with a subset of tegument proteins (182), like pUL46, pUL47, and pUL49 for the alphaherpesviruses, are incorporated (156). Two conserved proteins playing major roles in this process, glycoprotein M and pUL11, have been identified. Glycoprotein M, an envelope protein of matured virion, helps in retrieving envelope glycoproteins from the cell surface and retaining them at the envelopment site (183), while pUL11, a small myristoylated protein, directs envelope protein to future envelope sites (184, 185). At this stage, complex associations between many different tegument proteins, between tegument proteins and the nucleocapsid, and between tegument proteins and the cytoplasmic tails of various conserved and non-conserved envelope proteins (186, 187) are required for the ultimate assembly of a mature herpes virion (188). Following secondary envelopment, a mature herpesviral particle within a cellular vesicle is formed and transported to the plasma membrane (156), by anterograde cellular microtubule-dependent molecular motor kinesin (189). The newly produced virion particles are then released into the extracellular spaces following the fusion of vesicles with the plasma membrane (190).

1.10 Establishment of latency

EHV-1, like other herpesviruses, can establish a lifelong presence within cells of a susceptible host following primary infection. The initial stages of EHV-1 infection of the upper respiratory epithelial tract (URT) are accompanied by
progression into a stage of latency in which infected horses show no clinical
signs of the disease, virus shedding, or cell-associated viremia (77, 94). While
productive infection by EHV-1 leads to active viral gene expression in a well-
coordinated manner as described above, the hallmark of latency is the restriction
of viral gene expression which culminates in failure to synthesize viral factors and
absence of infectious virus particles. The primary site of latency establishment by
EHV-1 in the horse has been a subject of debate. While some studies have
demonstrated that latency of EHV-1 occurs in lymphocytes, both circulating and
those in draining lymph nodes (191-193), others have shown that the sensory
nerve cell bodies within the trigeminal ganglia are the preferred primary site of
latency for EHV-1 (13, 194). While about 80% of CD5+/CD8+ T- lymphocyte
population have been demonstrated as the predominant site of latency in
lymphoid cells, a smaller sub-population of 20% CD5+/CD8-/CD4- cells have also
been found to support latency of EHV-1 (195). Regardless of the site of
establishment, it appears that the ability of EHV-1 to pass into a latency stage is
a deliberate biological behavior that the virus utilizes to perpetuate itself in the
host and this enables viral spread to susceptible horses upon reactivation. During
latency, the expression of the EHV-1 genome is repressed and only the latency-
associated transcripts (LATs) antisense to either the immediate-early viral gene
(ORF 64) or a regulatory early gene (ORF 63) are present in infected cells (94,
193, 194). The exact molecular and physiological mechanisms that direct latency
in EHV-1 infected horses are poorly understood. However, latency has been
much better studied in HSV-1 and findings reveal that the major detectable
transcript lies within an 8.6-kb sequence antisense to and overlapping the immediate-early (IE) gene IE-1 (ICP0) (193). The LAT gene itself is a 2.0 kb transcript lacking a polyadenylation site and found mostly in the nuclei of infected neurons (193). In HSV, LAT can promote latency but is dispensable for maintenance or viral reactivation from latency (196).

It has been reported that reactivation of latent EHV-1 is possible following exposure to stressful conditions such as transportation, handling, re-housing, and weaning or following the administration of corticosteroids (197-199). The fact that EHV-1 has been experimentally reactivated from cases of natural infection and in experimentally infected cases following administration of immunosuppressant (13, 192, 199) suggests that horses harboring latent EHV-1 could periodically shed the virus following exposure to stressors. Also, viral factors such as a defective thymidine kinase in several alphaherpesviruses, including EHV-1, have been shown to cause impaired ability to reactivate from latency following experimental infection (197, 200-202).

As a result, the cycle of persistent latent infection followed by reactivation of the virus with shedding into nasal mucus may enable virus propagation and disease spread to susceptible uninfected horses. In certain instances, the characteristic respiratory illness followed by nasal shedding is absent following EHV-1 reactivation and such horses are therefore silent virus shedders (199). It has been reported that during the reactivation process a small fraction of lymphocytes carrying the latent EHV-1 genome can progress towards active transcription resulting in DNA revival and fusogenic viral glycoprotein expression.
on their cell surfaces ultimately leading to active virus replication (197, 199). The fine details of the molecular mechanism underlying reactivation of EHV-1 from its quiescent state to a lytic productive infection remain elusive. However, it has been suggested that the IE gene promoter of a latent EHV-1 can be trans-activated by the presence of another equine herpesvirus, EHV-2, in a mixed infection (203).

1.11 The Economic importance of EHV-1 to the US horse industry

About 10 million of the world horse population resides in the United States (204). The horse industry is a large and economically diverse industry which accommodates a wide array of economic activities. It has been reported that in the US, the horse industry generates annually an income of about $102 billion when considering both direct and indirect spending (205). With such enormous revenue generated from the horse industry in the US, an outbreak of any disease affecting its horse population is likely to perturb the economic health of the industry. The relevant effects of EHV-1 on the equine industry have been summarized (109). Firstly, EHV-1 outbreaks may result in cases of subclinical to mild respiratory illness especially with young athletic horses developing pyrexia and thus leading to interruptions of training programs. This is considered the least important economic effect of EHV-1 disease. Secondly, the incidence of abortion during the third trimester of gestation in pregnant mares results in major losses to the growth of the industry. Thirdly, outbreaks of the neurologic form of the disease, EHM, are very severe and may lead to deaths of horses, disruption of breeding or training schedules, cancelation of horse events, and extensive
movement restrictions with consequent management difficulties at racetracks, training centers, and other horse events. Even though horses may recover from the disease, their productivity is usually compromised and huge sums of money are expended in the care and management of horses infected with EHV-1.

1.12 Pathogenesis and disease manifestations

The pathogenesis of EHV-1 infection has been described by the study of an experimental model of infection using the EHV-1 strain, AB4 (94). EHV-1 is a highly contagious viral pathogen of horses usually transmitted following direct contact with infectious materials such as nasal discharges and materials from aborted fetuses or indirectly by fomites (77). In horses lacking protective mucosal immunity, nasal and mucosal epithelial cells are the primary sites of replication of EHV-1 (206, 207). Subsequently, virus replication is quickly followed by erosions of epithelial cells of the URT due to necrosis and inflammatory cellular responses which ultimately lead to nasal shedding of infectious virus (94). Once in the URT, EHV-1 can spread quickly utilizing and hijacking infected mucosal monocytes to invade the deeper connective tissues (208, 209). As a result, EHV-1 can cross the basement membrane, infecting leucocytes in the lamina propria and endothelial cells of blood and lymphatic organs (209). Within 24 h of infection, infected mononuclear leucocytes could be found present in the sinuses and parenchyma of respiratory tract-associated lymph nodes (207). Here, EHV-1 undergoes a second round of replication and viral particles are significantly amplified culminating in infected leucocytes escaping, via the efferent lymph, into the blood-vascular circulation leading to a state of cell-associated viremia (206,
The ability to establish viremia is key and defines the outcome of EHV-1 pathogenesis produced from the second round of replication. Viremia facilitates the dissemination of the virus to tertiary replication sites in the endothelium of the pregnant uterus or the central nervous system (77) leading to two clinically important sequelae of EHV-1 respiratory infection, namely abortion or a neurological syndrome (197, 213).

1.12.1 Respiratory disease

Equine herpesvirus type 1 (EHV-1) is one of the leading causative agents of equine respiratory disease, an infection of the upper airway mucosal epithelium seen primarily in young horses. The virus is highly ubiquitous among horse populations causing an epidemic disease early in life with an estimated 80 - 90% of horses being infected by 2 years of age (7). Following contact with an infectious viral particle, the mucosal epithelial cells of the URT of an infected horse are the prime target of EHV-1 where the virus undergoes its first round of replication (Fig. 1.5). Within 12 h post-infection, progeny virus and viral antigen are detectable in the respiratory epithelium of an infected horse (94) and the virus can quickly spread to the respiratory endothelium within 24 hours of infection (214). Besides, leucocytes in the adjacent lamina propria and endothelial cells of blood and lymphatic vessels are also infected due to a cell-to-cell spread of the infectious virus from the respiratory epithelium facilitating viral spread throughout the body (94, 215).

Subsequently, erosions due to epithelial cell necrosis and an acute inflammatory response may occur usually within the first week of the respiratory
disease leading to shedding of infectious virus particles (94). Depending on the pathogenicity of the EHV-1 strain, the incubation period of infection may either be short (one to three days) (216-218) or prolonged (up to ten days) (79). EHV-1 primarily results in respiratory tract disease (rhino-pharyngitis and tracheo-bronchitis) (219) presenting a clinical picture similar to other viral respiratory pathogens of the horse (e.g., equine influenza virus, adenovirus, rhinovirus, or arteritis virus) (7). Although a majority of such respiratory infections run a subclinical or mild course, and a large number of foals seroconvert without manifesting clinical signs, there may be visible signs of nasal discharge and coughing in some young naively exposed horses (77, 219). Previously exposed horses have immune memory that helps reduce the clinical severity of the disease and are infected for only a short duration (7, 207, 214). Depending on the age and the immune status of the infected horse, the respiratory infection may be mild in older horses, pregnant mares, and previously exposed horses even following virus reactivation from latency (77). Experimental infection using the virulent Ab4 strain of EHV-1 revealed a biphasic pattern of pyrexia which may last for up to ten days (216-218). The clinical picture of the disease includes moderate depression and anorexia, conjunctivitis and serous ocular discharge, and notably a serous nasal discharge which rapidly progresses to mucoid and mucopurulent discharge (77). The presence of mucopurulent discharge can be associated with a secondary bacterial infection which may exacerbate the disease. There is progressive lymphadenopathy mainly affecting submandibular lymph node (LN) (77) and evidence of leukopenia (both lymphopenia and
neutrophilia) have also been reported (6, 220, 221). Occasionally, retropharyngeal LN may also be enlarged and become palpable for some days (77). Lymph nodes may reach maximum size between three to ten days and may remain enlarged for several weeks following infection (77). In some infected foals, EHV-1 may reach the lungs inducing bronchopneumonia as a result (83).

While infected horses may occasionally cough, the severity and duration of clinical signs of the disease in a horse are influenced by proper hygiene and rest from exercise or training (222). Generally, the upper respiratory tract disease (URTD) associated with infection by EHV-1 is short-lived and of acute course with clinical signs and virus shedding manifesting for the first few days following infection (7). Although the prognosis of URTD from EHV-1 is good with spontaneous recovery by the end of the second week of onset of infection, severe secondary bacterial infections can prolong the illness and compromise the prognostic outlook for survival (7). Upon recovery from URTD caused by EHV-1, some horses may develop non-specific bronchial hypersensitivity, resembling chronic obstructive pulmonary disease, which may hinder their performance and lead to poor performance syndrome (222).

1.12.2 Abortion, neonatal and perinatal disease

The potential health consequences of EHV-1 extend beyond URTD and the virus may invade other organs causing more pronounced disease manifestations (7). One of the sequelae of EHV-1 URTD is abortion in which the virus travels to distant sites such as the reproductive tract by cell-associated viremia or latent viral reactivation (223) thereby inducing premature detachment
of the fetus from the placenta, stillbirth, or weak neonatal foals (224). Pregnant mares infected with the virus may abort spontaneously without prior signs of primary URTD by EHV-1 (53, 225-228). The important roles exerted by host immune and inflammatory responses, and vascular coagulation cascades mediating EHV-1-induced abortion have not been fully elucidated (77). However, EHV-1 infection of the endothelial cells of a pregnant uterus results in vasculitis that particularly affects the small arteriolar networks of the glandular layer of the endothelium at the base of microcotyledons (228-231). Within 9 to 13 days post-infection, endothelial cell infection becomes widespread resulting in multifocal vasculitis of the affected blood vessels (77). The appearance of microthrombosis within blood vessels may sometimes promote thrombo-ischemic necrosis of the cotyledons and intercotyledonary stroma causing the fetus to detach from the placenta (231). The aborted fetus dies from anoxia during a rapidly progressive placenta-endometrium separation that immediately precedes the event of expulsion (7). Widespread vascular endothelial damage may cause the fetus to be aborted even before any detectable level of virus is transferred via the placenta to the fetus (228).

Experimentally induced abortions by EHV-1 in which virus was not recovered from the aborted fetus have been reported to be as a result of either maternal stress or pyrexia (232, 233). In another experimental study, the extent of uterine vasculitis and intercotyledonary necrosis corresponds to reduced viral burden in the aborted fetus with fewer lesions found in mares aborting virus-positive fetuses (231, 234). The severity of the disease (abortion) usually
depends on some factors such as the pathogenicity of the EHV-1 strain involved, the level and magnitude of viremia, and the hormonal state of the pregnant mare. More virulent strains of EHV-1 such as Ab4 have been reported to produce more pathologies including abortion at a higher rate in pregnant mares than the less virulent strains like V592 (213). The pathogenesis of EHV-1-induced abortion by the less virulent strains of EHV-1 is not clear but it appears that those strains have reduced affinity for endothelial cell invasion (77). It has also been reported that the magnitude rather than the duration of viremia is a significant correlate of abortion induced by EHV-1 during an experimental challenge (213). Similarly, hormones such as prostaglandin and chorionic gonadotrophin (CG) released by the placenta have been reported to exert some roles in reactivating the virus and initiating abortion (77, 94). EHV-1 may be transferred by the placenta to the fetus thereby inducing multi-organ pathologies. EHV-1-infected fetuses born alive become sick either at birth or within one to two days of parturition (235-238). However, such foals do not live long because they consequently die as a result of the rapid deterioration of their health (94). Infected foals show severe respiratory distress that amplifies the risk of viral pneumonia or secondary bacterial infection, which lead to respiratory failure within a few days (83, 239). EHV-1 infected foals may also display signs of gastrointestinal disease (manifested in excreting watery diarrhea) and neurological signs such as visual and vestibular defects (236). Prognosis is grave and no treatment is available to stop the fatal clinical deterioration of health in infected foals. It has been reported
that congenital EHV-1 infection can be epizootic and may occur in association with an outbreak of EHV-1 abortion (7).

1.12.3 Myeloencephalopathy

Another clinical sequel of EHV-1 respiratory disease is the neurological form of the disease termed equine herpesviral myeloencephalopathy (EHM), sometimes appearing after one week of infection (83, 240-242). Neurologic symptoms may be simultaneously present with respiratory illness or abortion or may occur independently (243, 244). Fundamentally important in the spread of EHV-1 is cell-associated viremia which effectively disseminates the virus to the vasculature of the CNS. The immunologically privileged intracellular location of the virus appears to protect it from inactivation by circulating antibody and permits dissemination to other tissues, including the CNS, even in the presence of high levels of antibody (245). Similar to other herpesviruses, EHV-1 is capable of a direct cell-to-cell spread without establishing an extracellular phase (246). The vascular endothelium serves as the initial site of infection in the CNS and appears to be the preferred site for replication of EHV-1 after the transfer of the virus from circulating leukocytes (241). The infection of the endothelial cells and the accompanying inflammation of the vasculature of the CNS is central to the neurological syndrome caused by EHV-1 (206, 210, 230, 247). The vasculitis of the endothelium caused by EHV-1 may be as a result of two different mechanisms; the first being the direct damage of the endothelial lining of the blood vessels during EHV-1 replication and the second, a result of immune complex formation between EHV-1 and antibody (Arthus-type reaction) (224).
The development of vasculitis with or without hemorrhage and thrombo ischemic-necrosis of small blood vessels in the brain or CNS is a common feature of the neurological form of EHV-1 (77). The observed clinical signs in infected horses are a culmination of the vasculitis, hemorrhage, edema, necrosis, and ischemia that can result from the virus having a profound predilection for the vascular endothelium (224). Indeed, the ability of certain EHV-1 strains to inflict damage on the CNS is not reflective of their neurotropism but rather a marked endotheliotropism (210, 229, 230, 247, 248). However, the finding of chorioretinopathy and neural lesions in some experimentally infected, specific pathogen-free ponies suggests that at least some strains of EHV-1 may exhibit neurotropism (249). There seems to be no satisfactory scientific explanation for the variable incidence of EHM and different clinical manifestations observed during outbreaks of EHV-1 (238, 250). Several factors including age, sex, reproductive status (including the stage of pregnancy), immune status of the horse, the magnitude of the challenge, strain variations, and perhaps the route of infection influence the clinical outcome of EHV-1 infection (238, 250, 251).

Clinical signs of the neurologic disease may become apparent within 2 weeks of URTD or may occur without any antecedent sign of the disease (241). Clinical signs are highly variable and widespread depending on the location of the neurologic lesions and usually peak between two to three days of onset (77). Generally, there is fever, inappetence, distal limb edema, abortion, fetal death, or neurologic disease which are usually variable in different groups of horses on a particular farm (238). The extent of neurological dysfunction ranges from...
temporary ataxia with an abnormal gait to complete paralysis. Conscious proprioceptive deficits have also been observed (77). The neurological disorders affect mainly the hind limbs, although complete recumbency or tetraplegia have also been observed (77, 238, 252). In some cases, there are signs of bladder dysfunction with accompanying urinary incontinence and scalding of the perineal area or urinary retention which may lead to colic (253). The prognosis for non-recumbent horses is favorable but not so much for recumbent horses which may develop complications such as pneumonia, colic or bladder rupture (77, 238, 254) and are generally euthanized.

1.12.3.1 *Recent Outbreaks of EHM*

Outbreaks of EHM, among domestic horse populations, have been recorded for centuries. Today, a resurgence in the number of EHM cases across the world has necessitated the classification of this syndrome as an emerging disease of the horse. According to the Center for Emerging Issues report of 2007, EHM met the requirement for an emerging infectious disease based on (1) the more virulent nature of the circulating EHV-1 strains than previously reported and (2) increased incidence of the disease with heightened case fatality rate (255). Increased outbreaks of EHM were reported not only in North America and Europe, but also in Africa, Oceania, and Asia (256-266). The recent increased incidence of EHM during EHV-1 outbreaks supports the observation that the currently circulating neuropathogenic EHV-1 strain has evolved into a more virulent strain producing higher morbidity and mortality than in the past (267). EHM has been associated with an $A_{2254} \rightarrow G_{2254}$ mutation in the viral DNA
polymerase (ORF30). Generally, neuropathogenic strains have aspartic acid at position 752 whereas non-neuropathogenic strains possess asparagine (268, 269). In field outbreaks, this association is strong but not absolute, and there are other factors that could contribute to neuropathogenicity (109, 270).

Approximately, 14 percent to 24 percent of EHV-1 isolates from horses with EHM lack this neurological marker suggesting that the so-called non-neuropathogenic genotype of EHV-1 can also cause EHM (271, 272). This disease condition is a major concern for the horse industry considering its negative impact on the economic health of the industry.

The associated risk factors for this increased incidence of EHM are still poorly defined. However, outbreaks have been reported mostly at places such as riding schools, racetracks, and veterinary hospitals where horses from different origins congregate (256, 262, 273). The high stocking density of stabled horses during events such as horse racing may facilitate the quick spread of EHM by direct contact when outbreaks occur. International movement of horses has also played a role in some recent outbreaks of EHM (267, 274). Other factors that have been reported to facilitate increased incidence of EHM include poor biosecurity measures and presence of stressors (256, 275) along with other ill-defined environmental and host factors (276). Finally, the mutant EHV-1 (G\textsubscript{2254}) is now widely distributed within horse populations which would tend towards the increased incidence and severity of recent EHM outbreaks.
1.12.4 Ocular disease

Occasionally and particularly in foals, respiratory tract infection with hypervirulent strains of EHV-1 is accompanied by severe ocular disease such as uveitis or chorioretinitis (277). Within three to five weeks of URTD by EHV-1, foals may develop three distinct types of chorioretinal lesions (focal, multifocal or diffuse) without uveitis (249). Although the first report of EHV-1 associated chorioretinitis was in llamas and alpacas (278, 279), the disease condition has also been reported in natural outbreaks of paralytic EHV-1 infection involving a mare and foal (250). More recently, an incidence rate of 50-90% of horses was shown to develop chorioretinal lesions during an experimental challenge with EHV-1 (280). Similar to the pathogenesis of EHV-1 induced abortion and neurologic syndromes, replication of EHV-1 in the vasculature of the chorioretina may result in ischemic necrosis resulting in visual impairment (281). Apart from chorioretinitis, uveitis is another ocular condition seen in some foals following outbreaks of EHM in mares and stallions (238). Young foals that come in close contact with EHM-infected mares and stallions are at high risk of developing ocular disease associated with EHV-1 (238).

1.13 Laboratory diagnosis

A rapid diagnosis of URTD associated with EHV-1 within a group of horses is highly desirable to aid therapeutic decisions and shape future control strategies to prevent an epidemic outbreak of the disease (7). Usually, the presenting clinical sign alone is not sufficient to reach a precise diagnosis as the initial clinical presentation may also resemble that of equine influenza,
adenovirus, etc. As a result, laboratory diagnostic confirmation of EHV-1 induced URTD is predicated on the ability to isolate the virus from submitted clinical materials. Polymerase chain reaction (PCR) is a useful diagnostic tool for rapid identification and detection of genomic materials of EHV-1 in clinical or pathological specimens such as aborted fetus, placenta, nasal swabs, nasal discharges, brain and spinal cord, paraffin-embedded archival tissues, and infected cell cultures (282-286). Perhaps the most sensitive diagnostic tool for EHM is the RT-PCR which can discriminate isolates possessing single nucleotide polymorphism (SNP) in the ORF30 gene associated with the neurologic phenotype of the disease (287). One major caveat to the use of PCR is that it is not able to distinguish nucleic acid from a viable virus from that of a non-viable virus. It has been reported that the agreement between PCR and virus isolation is about 85-90 percent (77). This may be a particular concern when interpreting the presence of extremely low levels of viral DNA in clinical samples.

Direct detection of viral antigen from clinical samples using immunofluorescence staining also provides for rapid diagnosis of EHV-1. Cells from nasopharyngeal secretions can be stained with fluorescent antibodies against viral antigens for demonstration of a positive EHV-1 disease outbreak (77, 219). However, it is also important to confirm such results by virus isolation from submitted clinical specimens. Using type-specific antigen, Serological diagnostic tests such as virus neutralization (VN), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA) can also be employed on paired serum samples to differentiate EHV-1 from EHV-4. Immunohistochemically,
EHV-1 antigens can be detected in paraffin-embedded tissues from infected horses using immunoperoxidase staining (77). Histopathological examination of paraffin-embedded tissue sections can also be employed to identify pathognomonic lesions typical of EHV-1 infections.

The gold standard technique for laboratory diagnosis of EHV-1 is virus inoculation of cell cultures for isolation of the virus. The virus can be isolated from a variety of cell lines including those derived from the horse (EEC), rabbit (RK-13), monkey (Vero), and cattle (MDBK) (77, 152). The cytopathic effect (CPE), develops rapidly in cell cultures as clusters of rapidly enlarging, rounded, and detached cells which are characteristically herpetic in appearance (77). Virus culture and isolation should be carried out concurrently with rapid diagnostic testing (PCR), to retrospectively be able to biologically and molecularly characterize the virus isolate. Confirmatory diagnosis of EHV-1 should rule out other differentials such as EHV-4, influenza virus, adenovirus, rhinovirus, equine arteritis virus, and Sarcocystis neurona infection which may all present disease phenotypes that mimic EHV-1 infection.

1.14 Current treatment and control recommendations

There is no specific drug effective against EHV-1 disease conditions. However, good hygiene and management practices together with symptomatic treatment of infected horses may help curtail the spread of the viral infection. The current recommendations for treatment of horses include offering supportive care in cases of recumbency, nutritional care and hydration, frequent bladder and rectal evacuation to prevent colic, and reduction of CNS inflammation (288, 289).
Symptomatic treatment with non-steroidal anti-inflammatory agents as an adjunct therapy may be helpful (109, 224). Similarly, corticosteroids and immunomodulatory agents may be used to symptomatically treat early signs in cases of EHM. However, there is no evidence-based study to support the effectiveness of either drug class and caution must be applied not to reactivate virus shedding in latently infected horses (109, 152). Corticosteroids are thought to be protective against the cellular response to the infection of CNS thereby preventing the development of vasculitis, hemorrhage, thrombosis, and edema that are prominent early lesions of EHV-1 myeloencephalopathy, and their use is only reserved for severe cases of EHM (109).

Similarly, the administration of immunostimulants before horses are exposed to stressors could help prevent viral reactivation and replication but their value for treating EHV-1 infection is yet to be ascertained (109). Antiviral drugs especially virustatic agents like acyclovir derivatives are of theoretical value for the treatment of EHV-1 infection as they demonstrated in vitro efficacy (290). Beside acyclovir, prophylactic administration of valacyclovir hydrochloride has been tried in experimentally infected horses with demonstrable benefits (291). Ganciclovir has been demonstrated to be the most potent inhibitor of EHV-1 infection in an in vitro study that investigated the efficacy of many antivirals against EHV-1 (290), and in a more recent study, it also offers a much-improved bioavailability, in vivo (292) compared to acyclovir.

EHV-1 infection, as with other herpesviruses, is more complicated than most other viral infections; the ability of the virus to establish persistent latent
infection ensures that the virus is naturally maintained in horse populations all year-round. Also, EHV-1 has evolved a plethora of strategies to evade many components of the host innate and adaptive immune responses (293). As a result, an efficient EHV-1 vaccine must be able to invoke strong and sustained levels of humoral and cell-mediated immunity against the virus. In addition, since the establishment of cell-associated viremia is a prerequisite for the development of abortion and EHM, an effective vaccine candidate must, also, be able to stimulate those immune responses needed to block the development of cell-associated viremia. The currently available commercial vaccines against EHV-1 in North America are in the form of modified live vaccine (MLV) and inactivated whole virus vaccine. Both the Rhinomune (Boehringer Ingleheim), an MLV, and Pneumabort K-1B (Zoetis), an inactivated vaccine, reduced the clinical incidence of disease in an EHV-1 vaccine challenge study with the former offering better protection (294). However, the effectiveness of either vaccine in preventing EHV-1 induced abortion or EHM is still far from proven. EHV-1 antigen is also incorporated in some multivalent vaccines marketed across the US in their inactivated forms. Recombinant vaccine models expressing EHV-1 gB, gC, and gD reduced the initial nasal viral shedding in vaccinates but offered less protection against cell-associated viremia and clinical signs of disease (295, 296). Intriguingly, a recombinant vaccine expressing EHV-1 IE significantly reduced cell-associated viremia in vaccinated ponies however, its effect on EHV-1 induced abortion and EHM remains inconclusive (297). There is currently no available vaccine that completely prevents EHV-1 infection, or the development
of viremia or the establishment of latency, and EHM has been observed in vaccinated horses (252, 289, 298, 299). Despite that, it is recommended that all horses at risk of EHV-1 exposure should be vaccinated to help reduce the severity of clinical diseases associated with EHV-1. The updated version of AAEP guidelines for vaccination for adult horses provides a detailed recommendation for vaccinating against EHV-1 (300).

Control measures for curtailing EHV-1 infection are aimed at reducing the spread of the virus to susceptible horses and also at preventing the reactivation of the virus in latently infected horses (79, 246, 301). Infected sick horses are primary sources of infectious EHV-1, and as such, should be isolated from the rest of the herd to prevent direct contact with un-infected horses. Also, infected materials such as aborted fetus, placenta and uterine fluids from mares that have aborted should be disposed of appropriately to curtail the spread of EHV-1 (224). High-level biosecurity measures should be put in place in farms and all visitors should be encouraged to use a footbath and wash their hands before entering or leaving horse farms. Infected equipment must be disinfected or disposed of, and separate equipment and personnel should be engaged to work on affected and unaffected horses to prevent horizontal transmission of the disease (246). Movement of horses and visitors should be restricted onto and off the infected farm premises until laboratory tests indicate negative results for EHV-1 infection. Newly acquired horses should be quarantined from the rest of the herd for at least three weeks and must be certified negative for EHV-1 before being allowed to join the resident population. Horse owners and horse farmers should
immediately report EHM outbreaks to relevant government agencies to contain
the spread of the disease and to help formulate policies against future outbreaks.

1.15 The type-I IFN system

Mammals are constantly exposed to a wide array of pathogens and have
evolved a systematic approach to remain protected in most instances against
invading pathogens such as viruses. As the specific adaptive arm of the immune
response requires days to several weeks to become fully activated, the non-
specific innate arm plays a central role in immediately combating viral invaders
(302). In the absence of pre-existing adaptive immunity, the innate immune
function of the host immune response is immediately triggered to recognize
incoming viruses and curtail their replication and spread to uninfected cells (302).
The first line of defense against viral pathogens in a mammalian host consists of
a soluble class of cytokines called interferon (303). IFN was first described in the
early 1950s as an important host factor that is rapidly produced in virus-infected
cells and whose release enables protection of neighboring cells against virally
induced cell-to-cell spread (304). To date, three types of IFN, namely type-I,
type-II, and type-III, have been recognized to be produced by different cell types,
bind different receptors, and mediate different biological functions (15, 305).
Although structurally unrelated and operating through distinct receptors, these
cytokines are classified as IFNs on the basis of their partially overlapping
activities (306). The type-I IFN of mammals comprises a large group of
molecules, but only eight classes have been recognized in horses to date: IFN-α,
IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-ω, IFN-ν, and IFN-μ (307). They are produced by
different types of host cells including epithelial, fibroblast, and dendritic cells to mediate distinct roles during viral infection, cell growth, and pregnancy. While IFN-α/β, hereafter referred to as the type-I IFN, are virally induced cytokines, IFN-κ, ε, δ, and ω act as regulators of maternal recognition of pregnancy (15).

Type-I IFN induction by virus-infected cells occurs in 3 phases—sensitization, induction and amplification (302). In the immediate early or sensitization phase (Fig. 1.6.A.), innate immune sensors known as pattern recognition receptors (PRRs) interact with conserved viral motifs resulting in the coordinate activation of cellular transcription factors such as nuclear factor-κB (NF-κB), interferon-regulatory factor 3 (IRF3), IRF1, and ATF-2/c-Jun. Activated transcription factors collectively called the ‘enhanceasome’ then induce the production of low levels of IFN-β (23, 306, 308, 309). The mediators of viral recognition that lead to the production of IFN-β consist of a group of PRR located either in the cytoplasm or in the transmembrane of cell surface/endosomes; areas that allow these receptors to efficiently detect viral invasion. These receptors include toll-like receptors (TLRs) which recognize a variety of viral structures, retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) family which recognizes intracellular dsRNA, and the recently identified cytosolic DNA sensor-DNA-dependent activator of IFN-regulatory factors (DAI) receptor (310-312).

Following ligand binding, PRRs recruit and interact with the appropriate adaptor protein which plays an essential role by phosphorylating and activating several constitutively expressed transcription factors for a low-level IFN-β transcription. While TLRs majorly interact with Toll/IL-1 receptor (TIR) containing adapters
such as MyD88, TIR-domain-containing adapter-inducing IFN-β (TRIF), Mal and TRIF related adaptor molecule (TRAM), RLRs interact with either stimulator of interferon genes (STING) or mitochondrial antiviral signaling protein (303, 313). In resting cells, NF-κB is constitutively present in the cytoplasm in its inactive state, forming a complex with its inhibitor IκBα (314). Following PRR activation, IκBα becomes phosphorylated (mediated by I-kappa-B kinases (IKK) recruited by the adaptor proteins) and undergoes subsequent degradation thereby releasing NF-κB to translocate to the nucleus and induce target genes (303). Similarly, IRF3, which is also constitutively expressed in resting cells, is phosphorylated by IKK-related kinases IKKe and TBK-1 upon pathogen-associated molecular pattern (PAMP)-PRR interaction causing IRF3 to homodimerize and undergo nuclear translocation (303). There, they could either interact with histone acetyl transferases such as CREB-binding protein (CBP) and p300, initiating their immediate association with IFN-β promoter (303), or they could directly activate a subset of ISGs even in the absence of IFN (315, 316). Not much is known about the roles of ATF-2/c-Jun, but it is suggested that the complex is inherently intranuclear even in its inactive form, and is stimulated by phosphorylation of its activation domain (317).

In the subsequent early or inductive phase (Fig. 1.6.B.), secreted IFN-β binds to its cognate receptors present ubiquitously on cell surfaces initiating IFN signaling. The major components of this cross-talk leading to type-I IFN signaling include two receptor subunits, two Janus-activated kinases (JAKs), two signal transducers and activation of transcription (STATs) and the IRF- family
transcription factor IRF9. This signaling pathway can be summarized in five main
steps (27): (a) the extracellular IFN-driven dimerization of IFN-α/β receptor
(IFNAR) induces (b) phosphorylation-activation events in the cell, resulting in (c)
heterotrimeric, STAT-IRF, complex formation activating them for (d) nuclear
translocation where they (e) bind specific DNA sequences and stimulate
transcriptional processes. The receptor has two subunits: IFNAR1 and IFNAR2c
hereafter referred to as IFNAR2. These two subunits are required for a high-
affinity binding of secreted IFN-β as neither subunit alone binds effectively to IFN
(318, 319). Upon ligand binding, a signaling cascade commences with the
phosphorylation of TYK2 following its interaction with the cytoplasmic domain of
IFNAR1 (320, 321). JAK1 then interacts with IFNAR2 inducing the
phosphorylation and activation of TYK2 (322) which in turn cross-activate JAK1
to activate it further (27). A series of well-coordinated events lead to the
phosphorylation of IFNAR1, STAT2 and STAT1 in that order. Firstly, TYK2
phosphorylates IFNAR1 at tyrosine residue 466 (Y466) creating a docking site for
STAT2 (320). This is then followed by the phosphorylation of STAT2 at tyrosine
690 (Y690) by TYK2 creating a new docking site for STAT1 (323, 324). STAT1 is
subsequently phosphorylated at tyrosine 701 (Y701) by IFNAR2-bound JAK1
(325). Upon phosphorylation, STAT1 and STAT2 then dimerize and bind to IRF9
to form a heterotrimeric complex termed interferon stimulated gene factor 3
(ISGF3) (326). The newly formed heterotrimer then translocates to the nucleus
and binds to the promoter regions of ISGs (327), including IRF7, enhancing their
transcription (328). Activated IRF3 forms a heterodimer with IRF7, translocates
into the nucleus and binds to the promoter of IFN-α genes resulting in the production of IFN-α species as well as a greater number of ISGs (15, 302, 328).

In the delayed early or amplification phase (Fig. 1.6.C.), IFN-α/β further induce a greater range of ISGs and the protein products of these ISGs help restrict viral replication and spread leading to the establishment of a global antiviral state within the host cell (329).

1.15.1 ISG expression and function

ISGs have been described as the workhorse of the type-I IFN system (330, 331) whose antiviral effects block the transcription and translation of viral genes and also promote the apoptosis of virally infected cells (302, 332). The inhibitory effects of IFN on a wide range of mammalian DNA and RNA viruses have been described by using both in vitro and in vivo models (333-335). Prominent antiviral ISGs induced by IFN in virus-infected cells are protein kinase R (PKR), 2', 5'-oligoadenylate synthetase (OAS) and RNase L, RNA-specific adenosine deaminase (ADAR), and myxovirus-resistance (Mx) protein GTPases (330). It has been reported that dsRNA exert a major modulatory effect on protein phosphorylation, RNA editing, and RNA degradation that is catalyzed by actions of PKR kinase, OAS synthetase, and ADAR1 deaminase (336, 337). PKR is an intracellular sensor of dsRNA that is induced by IFN, and elicits an antiviral effect by inhibiting protein translation (338, 339). Although this enzyme is predominantly found closely associated with ribosomes in the cytoplasm of IFN-treated cells (340-342), small amounts of PKR has also been demonstrated by cell fractionation and immunofluorescence to be localized in the nucleus (342,
PKR contains several functional domains (344) such as the N-terminal domain that binds dsRNA and the C-terminal domain that possess kinase activities (329). Following dsRNA-mediated autophosphorylation of PKR (341, 345, 346), activated PKR catalyzes additional phosphorylation events of at least 6 proteins: the PKR protein itself (347, 348); the α subunit of eukaryotic initiation factor 2, eIF-2α (349); the transcription factor inhibitor IκB (350, 351); the Tat protein encoded by human immunodeficiency virus (HIV) (352); the 90-kDa NFAT protein (353); and the M-phase specific dsRNA-binding phosphoprotein MPP4 (354). Among the many substrates of PKR, eIF-2α remains the best characterized. Serine phosphorylation of eIF-2α, which occurs at position Ser-51 (349, 355), negatively regulates cellular pathways including primarily mRNA translation and protein synthesis (330) by impairing the eIF-2B-catalyzed guanine nucleotide exchange reaction (341, 345, 356, 357). Studies suggest that changes in IFN-inducible PKR phosphorylation play an important role in the antiviral actions of IFN and regulation of cell growth by IFN (341, 345, 358, 359).

In its inactive state, OAS is constitutively expressed in the cytoplasm of resting cells but becomes induced upon IFN treatment (360). It was initially thought that this enzyme primarily targets only the viral RNA for degradation as part of the antiviral IFN-mediated response, however, cellular RNAs have also been reported as targets of this pathway indicating its role in regulating cellular functions and apoptosis (329, 361). This pathway seems to be activated by dsRNA and requires two enzymes namely; OAS and RNase L for mRNA degradation (330). The synthesis of oligoadenylates (commonly referred to as 2-
5A) by OAS triggers the activation of RNase L, a latent endoribonuclease, by the binding of 2-5A oligoadenylates (330). A third enzymatic event involves the hydrolysis of oligonucleotides possessing 2', 5'-phosphodiester bonds, by phosphodiesterase thereby attenuating the 2-5A response (330). There is no structural homology between the dsRNA-binding domains of OAS and those of PKR and ADAR (362, 363), however, OAS, like PKR and ADAR (341, 345, 364), possesses separate subdomains for its dsRNA binding and catalytic activity (365, 366). Convincingly, this pathway has been demonstrated to regulate cellular processes and also restrict viral replication in in vitro and in vivo experiments (367-370).

The myxovirus-resistance (Mx) proteins were among the first IFN-inducible gene products to be studied in the context of a virus infection (371). Experimental evidence obtained from animal models revealed that Mx alone has the ability to restrict viral replication in the absence of any other IFN-α/β-inducible proteins (372, 373). Mx proteins are IFN-inducible GTPases and belong to the superfamily dynamin-like GTPases (374, 375). The intrinsic GTPase activity of Mx proteins indicates that their antiviral activity requires an enzymatic function (361). The highly conserved GTP binding activity is located within the N-terminal region of the protein (372-374). Mx proteins associate with themselves and importantly, with viral protein complexes mediated by the central and the C-terminal region of Mx (376, 377). The antiviral significance of Mx proteins was first demonstrated in influenza- and Thogoto-virus systems (361). The spectrum of antiviral activity and the underlying mechanisms of Mx proteins are dependent
on the phenotype of the Mx protein induced, its subcellular localization and the challenge virus (330). It has been demonstrated that MxA protein associates with the nucleocapsid of bunyaviruses promoting the redistribution of the viral capsid proteins in an attempt to inhibit bunyavirus replication (378). Similarly, the presence of Mx proteins either in the cytoplasm or nucleus of IFN-treated virus infected host cells determines whether viral nucleocapsid transport or viral RNA synthesis is blocked by Mx proteins (372, 374, 379, 380).

The functionality of both viral and cellular RNAs can be changed by posttranscriptional modifications such as deamination of adenosine to yield inosine enabling certain biological processes to be affected (381-384). ADAR1 deaminase is an example of an enzyme that is able to edit viral RNA transcripts and cellular pre-mRNAs (364, 385, 386). This enzyme was first identified to possess dsRNA-unwinding activity in Xenopus oocytes (387, 388) and it is known to catalyze deamination of adenosine to yield inosine in RNA substrates (330). The resultant effect of A to I transition creates an instability in the dsRNA helix by disrupting the base pairing making them become single stranded in character (387, 389). The catalytic domain of ADAR1 is situated in the C-terminal region of the deaminase (390-392) while the nucleic acid binding region is located in the N-terminal region of the catalytic domain (330). The biological significance of RNA editing by ADAR1 is in the potential to alter the protein coding capacity of the edited RNA transcript and sequence of replicated RNAs (330). Adenosine deamination is proposed as the mechanism underlying how RNA transcripts expressed early during polyomavirus infection are inactivated.
after viral replication (393). Similarly, an A to I editing at specific sites of neurotransmitter receptor pre-mRNAs by ADAR have been reported to alter receptor function (384, 392).

IFN can also shape the host’s immune response to infection by inducing nitric oxide synthase (iNOS2), and the MHC class I and II molecules (330). While nitric oxide produced by iNOS2 plays a key role in immunological defenses as an antimicrobial and antiviral agent, elevated MHC class I and II antigen levels mediated by IFN are believed to increase the efficiency of cellular immune responses to infections in the intact animal (330).

1.16 Viral evasion of type-I IFN response

Due to the importance of the type-I IFN system in restricting viral replication both in infected and uninfected cells, many viruses have adapted mechanisms to counteract the antiviral effect of the type-I IFN response. The mechanisms used by various viruses to inhibit the type-I interferon system involve: (1) mechanisms that interrupt the upstream mediators of viral recognition (394, 395) (2) mechanisms that circumvent the signaling pathways leading to interferon production (21, 31, 396), and (3) mechanisms that inhibit interferon-induced antiviral proteins (397).

1.16.1 Viral mechanisms that inhibit the upstream mediators of IFN production

Certain viral factors target PRRs in infected host cells to evade an early innate immune detection thereby escaping the antiviral effect of the type-I response during infection. For example, the US3 gene of HSV-1 interferes with TLR3-mediated signaling that enables the virus to evade type-I IFN response in
an infected host cell (398). Similarly, HSV-1 deletion mutants of the US3 gene express elevated levels of TLR3 and type-I IFN activity in infected monocytic cells when compared with the WT virus (399). In a different study, Schlender et al reported that respiratory syncytial virus (RSV) strain A2 and measles virus strain Schwarz block type-I IFN production in a plasmacytoid dendritic cell model mediated by inhibition of both TLR 7 and TLR 9 signaling (394). Multiple viral proteins have also evolved to specifically inhibit cytosolic viral sensors in order to avoid an immediate innate immune detection and activation. For instance, the US11 protein of HSV-1 binds to RIG-I and MDA-5 in order to inhibit their downstream signaling pathways, thereby preventing the production of IFN-β (395). Similarly, the nonstructural 1 (NS1) protein of the influenza A virus has been shown to inhibit the tripartite motif containing 25-caspase activation and recruitment domain (TRIM25-CARD) interaction which is required for the activation of RIG-I (400).

1.16.2 Mechanisms that circumvent the signaling pathways leading to IFN production

The two most common mechanisms employed by viruses to inhibit type-I IFN signaling are (1) virally derived proteins that inhibit the IRF3, IRF7, NF-κB, and the activator protein 1 (AP-1) signaling pathways and (2) virally derived proteins that inhibit the JAK-STAT signaling pathway. It has been shown that human papillomavirus encodes a protein, E6, that interacts with IRF3 affecting its ability to transactivate IFN-β induction (401). Recently, our laboratory demonstrated that the molecular mechanism for EHV-1 suppression of type-I IFN
induction involves interference with the IRF-3 signaling pathways (402). In a different study by Ciancanelli et al. the IRF-7 gene was shown to be defective in patients with severe susceptibility to H1N1 influenza, while susceptibility to other viral diseases such as CMV, RSV, and parainfluenza was unaffected (403). Different viruses have been reported to encode viral factors that targets different steps of JAK-STAT signaling pathway. The ICP 27 of HSV-1 has been shown to prevent phosphorylated-dependent activation and nuclear translocation of STAT1 molecules (404). Cellular expressions of V proteins from simian virus 5 and type II human parainfluenza virus have been reported to induce polyubiquitination of STAT1 and STAT2 targets (396). In another study, Human metapneumovirus decreases cellular levels of JAK1 and TYK2 by targeting them for proteasomal degradation leading to a consequential effect on IFN-β signaling (21). Our laboratory also reported that the molecular mechanism employed by EHV-1 to down-regulate type-I IFN production involves interference with the activation and nuclear translocation of STAT1 molecules in infected EECs (31). The important function played by the JAK and TYK2 kinases, and STAT proteins in antiviral response makes them attractive targets for viral proteins to evade type-I IFN biological effects.

1.16.3 Mechanisms that inhibit IFN-induced antiviral proteins

The end-products of type-I IFN signaling ensures that virally infected cells and bystander uninfected cells are protected from the damaging effects of viral infection. However, many viruses encode proteins that enables them to outmaneuver the global anti-viral effect of ISGs. The RING finger domain of
HSV-1 ICP0 has been reported to inhibit IRF3- and IRF7-mediated activation of ISGs (405). Similarly, it has been reported that dengue virus encodes some non-structural proteins, such as NS4B, NS2A, and NS4A, that down-regulate ISGs in human A549 cells (397). Yokota et al. also showed that herpes simplex virus induces suppressor of cytokine signaling 3, a negative regulator of JAK-STAT pathway, as early as 2 hpi in human amnion cells (Yokota et al., 2003). More recently, our laboratory reported that EHV-1 infection of EECs suppresses the expression of an IFN induced antiviral protein, viperin (‘virus inhibitory protein’, ‘endoplasmic reticulum-associated’, ‘interferon-inducible’) (406).

1.17 Statement of problems and hypothesis

EHV-I causes a disease condition worrying to many horse owners not only because of its tendency to affect reproduction but also because of its capability to progress to a neurological form, which affects horses’ well-being and their abilities to be utilized for sport and recreation. Outbreaks are costly for the equine industry-quarantines and tracing efforts funded by the government to curtail the spread of the disease amount to millions of dollars annually in the United States (407). In the same vein, horses are confined to stalls to prevent direct spread between horses and horse race events are canceled leading to a huge loss in revenue. The highly infectious nature of EHV-1 imposes an urgent need to develop a more effective vaccine than the ones presently available commercially. Additionally, there is no report of an effective vaccine against the neurological form of the disease.
Multicellular organisms possess sophisticated defense mechanisms, innate and adaptive immunity, that are immediately activated to counter the invasion of a wide array of pathogens within the body of a vertebrate host (408). However, successful pathogens such as EHV-1 have evolved a range of anti-immune strategies to overcome both innate and acquired immunity (31, 402, 406, 409-412), which play critical roles in their ability to cause disease. Over the years, several studies have investigated the underlying mechanisms employed by EHV-1 to counteract the adaptive immune arm of the host immunity. Unfortunately, studies investigating the impact of EHV-1 infection on the innate immune response of the host appear to be rudimentary. The type-I IFN system is a critical component of the host’s innate immunity against viral infection. It plays a dual role in the signaling pathway to preventing viral replication. Firstly, type-I IFN is responsible for inducing transcription of a large group of genes, which makes the host cells develop resistance to viral infections (15, 302, 328). Secondly, it can activate key components of both the innate and adaptive immune systems (413). Type-I IFN has applications in immunotherapy against viral infections and also in cancer treatment. Despite the recent increase in interest from researchers to decipher the intricate interaction between EHV-1 and the type-I IFN response, a lot of information is still missing. Characterization of type-I IFN response during EHV-1 infection using an appropriate experimental model will furnish explanations to questions on why the host is unable to induce a protective immunity against EHV-1.
Furthermore, a detailed knowledge of how the virus outsmarts the host type-I IFN system is highly fundamental in order to come up with the development of viable interventions that will limit the scourge of this infectious disease. Identifying certain critical steps in the virus replication cycle which are indispensable for its ability to infect host cells and dampen the host type-I IFN response will go a long way towards developing effective vaccines and therapeutic interventions against EHV-I infection. This is an ongoing line of research, and our study aims to expand prior knowledge on the underlying molecular mechanisms employed by EHV-1 to down-regulate type-I IFN response in host cells. The central hypothesis of this dissertation is that highly pathogenic EHV-1 and low pathogenic EHV-4 differentially alter type-I IFN signaling molecules. To test this hypothesis, the specific aims are:

(A) To investigate the relationship between neuropathogenicity of EHV-1 and suppression of IFN-α/β response.

(B) To investigate the effect of EHV-1 infection on signaling events leading to IFN-α/β response in vitro.

(C) To investigate the effect of EHV-4 infection on signaling events leading to IFN-α/β response in vitro.
### Table 1. Known equine herpesviruses

<table>
<thead>
<tr>
<th>Subfamily of Herpesviridae</th>
<th>Equus species</th>
<th>Gazella thomsoni</th>
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</thead>
<tbody>
<tr>
<td>Domestic horse (Equus caballus)</td>
<td>Donkey (Equus asinus)</td>
<td>Zebra (Equus grevyi)</td>
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</table>

**Alphaherpesvirinae:**

<table>
<thead>
<tr>
<th>a. Viscerotropic subgroup</th>
</tr>
</thead>
</table>
| Equine herpesvirus 1<sup>a</sup>  
*(Equid herpesvirus 1)<sup>b</sup>  
Equine herpesvirus 4  
*(Equid herpesvirus 4)* | Asinine herpesvirus 3  
*(Equid herpesvirus 8)* | Zebra herpesvirus isolates | Onager herpesvirus isolates |

<table>
<thead>
<tr>
<th>b. Dermatotropic subgroup</th>
</tr>
</thead>
</table>
| Equine herpesvirus 3  
*(Equid herpesvirus 3)* | Asinine herpesvirus 1  
*(Equid herpesvirus 6)* |

**Gammaherpesvirinae**

| Equine herpesvirus 2  
*(Equid herpesvirus 2)*  
Equine herpesvirus 5  
*(Equid herpesvirus 5)* | Asinine herpesvirus 2  
*(Equid herpesvirus 7)* |

Adapted from Allen et al. (77).

<sup>a</sup>Viruses located in the same horizontal row of the table represent closely related equid herpesviruses exhibiting minor genetic and antigenic divergence induced by natural adaptation of a common progenitor to different equid or animal species.

<sup>b</sup>Virus names in parentheses are nomenclatural designations assigned by the Herpesvirus Study Group of the International Committee on Taxonomy and Nomenclature of Viruses (ICTV) (414, 415). No ICTV designations have at this date been assigned by the Study Group to the zebra or onager herpesviruses (414). A neurotropic herpesvirus isolate from captive gazelle closely related to *Equid herpesvirus 1* has provisionally been designated *Equid herpesvirus 9* (80, 414, 416).
Table 1. 2 EHV-1 and EHV-4 gene products and their functions

<table>
<thead>
<tr>
<th>ORF #</th>
<th>EHV-1</th>
<th>EHV-4</th>
<th>Functional class of HSV homolog (core genes)</th>
<th>Gene products and proposed functions</th>
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**Nucleic acid metabolism**

**Envelope glycoprotein**
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<td>Tegument protein with molecular chaperone function</td>
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<td>Non-glycosylated membrane-associated protein, neuropathogenic virulence factor?</td>
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<td>UL31*</td>
<td>Nuclear matrix binding protein, interacts with UL34</td>
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<td>Associate with inner nuclear membrane</td>
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<td>UL36</td>
<td>ICP1/2, largest tegument protein, involved in both uncoating and egress</td>
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<td>UL37</td>
<td>ICP32, Tegument protein with nuclear export signal, involved in egress and virion maturation</td>
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<td>UL49.5*</td>
<td>Small membrane-associated protein</td>
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<td>Palmitoylated virion protein, associated with the Golgi</td>
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<td>ICP27, regulation of gene expression at the post-translational level</td>
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<td>UL9</td>
<td>Replication origin-binding protein</td>
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**Non-core essential genes**
Table 1. 5 EHV-1 and EHV-4 gene products and their functions (continued)

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<td>UL4*</td>
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<td>UL21*</td>
<td>UL23*</td>
<td>UL40*</td>
<td>UL41*</td>
<td>UL43*</td>
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<td>UL45*</td>
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<td></td>
<td>VP16</td>
<td>ICP4</td>
<td>gD</td>
<td>Nuclear phosphoprotein, involved in nucleolar localization</td>
<td>ICP0, promiscuous transactivator with E3 ubiquitin ligase domains involved in gene regulation</td>
<td>Nuclear protein, co-localized with UL3 and ICP22</td>
<td>Virion protein, essential for viral exocytosis</td>
<td>Tegument protein, associated with microtubules</td>
<td>ICP36, thymidine kinase (TK) required for nucleotide metabolism</td>
<td>Ribonucleotide reductase small subunit, involved in nucleotide metabolism</td>
<td>VHS, virion host shut-off protein, causes non-specific cellular mRNA degradation</td>
<td>Membrane-associated protein</td>
<td>gC, VP7.5, involved in cell attachment and adsorption, C3b-binding activity</td>
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<td>Virion protein, interacts with cytokeratin</td>
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<tr>
<td>US5*</td>
<td>gJ, protects from Fas-mediated apoptosis</td>
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<tr>
<td>US7*</td>
<td>gI, interacts with gE, involved in cell-to-cell spread</td>
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<tr>
<td>US8*</td>
<td>gE, forms complex with gI, Fc receptor activity, cell-to-cell spread</td>
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<td>US8.5*</td>
<td>Localized in nucleoli of infected cells</td>
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<td>US9*</td>
<td>Type-II membrane protein, involved in anterograde transport of envelope glycoprotein?</td>
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<td>US10*</td>
<td>Tegument protein, tightly associated with capsids</td>
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<td>US11*</td>
<td>Tegument protein, RNA-binding activity, and intercellular trafficking</td>
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Table 1. 7 EHV-1 and EHV-4 gene products and their functions (continued)

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Table 1.2 was prepared according to available data published in Allen et al (77), Nishiyama et al (417), Davison et al (146), Sarkar (418), Roizman (419), Kasem et al (420), Telford et al (81), Ma et al (421), and Ma et al (422). * indicates that the gene is dispensable for replication in cell culture; NA= Not available; † indicates conserved genes across all mammalian herpesviruses.
Figures.

Figure 1. 1  Schematic illustration of herpesvirus structure. The basic herpesviral structure consists of an envelope, a tightly woven dsDNA genome enclosed within the capsid, and a tegument layer. Several glycoproteins are present on the surface of the envelope.

Figure 1. 2  Lytic life cycle of EHV-1. The virus enters susceptible cells either by fusion at the cell membrane or by the non-classical endocytosis pathway. This is followed by the release of nucleocapsid into the cytoplasm of an infected cell. The nucleocapsid, which is transported to the nucleus via dynein, docks at the NPC and extrudes the viral DNA
directly into the nucleus. This initiates viral gene expression beginning with the transcription of IE (α) gene. Immediate early proteins are then synthesized in the cytoplasm and migrates to the nucleus where they direct the transcription of E (β) gene. Early protein, synthesized in the cytoplasm, translocates to the nucleus to initiate virus DNA replication and virus L (γ) gene expression. Next, some of the L proteins synthesized in the cytoplasm, migrate to the nucleus to form the capsid before encapsidation of the new virus DNA. The newly assembled virion then migrates through the nucleus and the cytoplasmic membranes before it is eventually released outside of the cell.

Figure 1. 3  Herpesvirus capsid formation. The basic assembly of a matured capsid consists of complex formation between major capsid protein and scaffold protein with the incorporation of a portal protein. An early step in the formation of a spherical procapsid (partial procapsids) involves complex interactions between the major capsid protein and the scaffold protein to produce what, first, looks like an angular segment. A complex of portal and scaffold protein is incorporated with the progressive enlargement of the partial procapsid. Once the procapsid is formed, virus DNA is packaged marking the exit of the scaffolding proteins creating a polyhedral shape.
Figure 1. 4  Herpesvirus egress pathway. Following intranuclear encapsidation of the virus genome, the herpesviral nucleocapsid will bud through the inner nuclear membrane resulting in perinuclear localization of an enveloped primary virion. This primary envelope becomes lost (de-envelopment) as the virus translocates into the cytosol where the nucleocapsid acquires tegument proteins. Final (secondary) envelopment then occurs in the cytoplasm derived from the TGN and the enveloped virion is transported in a vesicle to the plasma membrane for release.
Figure 1. 5 Schematic illustration of EHV-1 pathogenesis. EHV-1 primarily infects the respiratory epithelial cells. The virus is able to cross the basement membrane and invade the lamina propria where it infects circulating leucocytes. The virus then travels through the lymphatic system to regional lymph nodes where it undergoes amplification and infects peripheral blood mononuclear cells leading to a state of cell-associated viremia. The cell-associated viremia ensures that EHV-1 is disseminated to distant sites such as the endometrium of the pregnant uterus and the CNS causing inflammatory responses that culminate in the development of pathologies; abortion and EHM.
Figure 1.6 Virus-mediated IFN production. A. In the immediate early/sensitization phase, viral conserved motifs (PAMPs) interact with host innate immune sensors (PRR) leading to the production of low levels of IFN-β. B. In the early or inductive phase, released IFN-β bind to IFNAR1 and IFNAR2 triggering the activation of the JAK-STAT signaling pathway leading to the induction of ISGs including IRF7. With subsequent viral infection, activated IRF3 and IRF7 form heterodimers and translocate to the nucleus to upregulate the transcription of IFN-α species. C. In the delayed early or amplification phase, the synergistic effect of IFN-α and IFN-β induces enhanced transcription of a diverse set of ISGs creating a global antiviral environment. Illustration was based on Mossman and Ashkar (302).
CHAPTER 2: ABSENCE OF RELATIONSHIP BETWEEN TYPE-I INTERFERON SUPPRESSION AND NEUROPATHOGENICITY OF EHV-1.

Abstract

Equine herpesvirus-1 (EHV-1) infection is an important and highly prevalent disease in equine populations worldwide. Previously we have demonstrated that a neuropathogenic strain of EHV-1, T953, suppresses the host cell's antiviral type-I interferon (IFN) response in vitro. Whether or not this is unique to EHV-1 strains possessing the neuropathogenic genotype has been undetermined. Here, we examined whether there is any direct relationship between neuropathogenic genotype and the induced IFN-β response in equine endothelial cells (EECs) infected with 10 different strains of EHV-1. The extent of virus cell-to-cell spread following infection in EECs was also compared between the neuropathogenic and the non-neuropathogenic genotype of EHV-1. We then compared IFN-β and the total type-I IFN protein suppression between T953, an EHV-1 strain that is neuropathogenic and T445, an EHV-4 strain mainly associated only with respiratory disease. Data from our study revealed no relationship between the neuropathogenic genotype of EHV-1 and the induced IFN-β mRNA by the host cell. Results also indicate no statistically significant difference in plaque sizes of both genotypes of EHV-1 produced in EECs. However, while the T953 strain of EHV-1 was able to suppress IFN-β mRNA and type-I IFN biological activity at 12 hours post-infection (hpi), EHV-4 weakly induces both IFN-β mRNA and type-I IFN biological activity. This finding correlated with a statistically significant difference in the mean plaque sizes produced by the two EHV subtypes in EECs. Our data help illuminate how EHV-
1, irrespective of its genotype, evades the host cell’s innate immune response thereby enabling viral spread to susceptible cells.

Keywords: Neuropathogenic; Viral pathogenesis; Herpesvirus; Interferon; Equine; Genotype.

2.1 Introduction

EHV-1 infection is an important disease of equids that was first documented in the early 1930s (1). The viral infection is known for its clinical manifestations including respiratory disease, sporadic abortion during the third trimester of gestation, birth of weak newborns, chorioretinitis and neurological disease (11, 79, 105, 230, 423-425). Like most other herpesviruses, EHV-1 has the ability to establish latency after primary infection, leading to a carrier state in infected horses. Additionally, the disease is highly contagious among horses and one of the most devastating manifestations of EHV-1 infection is the neurologic form termed equine herpesvirus myeloencephalopathy (EHM). Although EHM is not new, there has been an increase in the outbreak of the disease condition since the year 2000 (105, 411, 426-428).

It has been suggested that distinct strains of EHV-1 differing in pathogenicity circulate in the field (272). Strains of EHV-1 have been broadly classified neuropathogenic and non-neuropathogenic, based on the presence of a single nucleotide polymorphism (A_{2254}→G_{2254}) in the viral DNA polymerase with G_{2254} associated with neuropathogenic strains (272). Generally, the EHV-1 strains possessing the neuropathogenic genotype are involved in neurologic outbreaks while those with the non-neuropathogenic genotype are predominantly
isolated from cases of sporadic abortions in pregnant mares (206, 210, 213, 239, 250). It is, therefore, noteworthy that some of the EHM cases are associated with the $A_{2254}$ non-neuropathogenic genotype, and the $G_{2254}$ neuropathogenic genotype does not necessarily lead to EHM (276, 429-432), indicating that other viral and host factors are involved in EHM.

Equine herpesvirus-4 (EHV-4) is a different virus type but closely related to EHV-1 with nucleotide sequence identity within individual homologous genes ranging from 55 to 84 percent and amino acid sequence identity ranging from 55 to 96 percent (81, 82). The virus was earlier classified as a subtype of EHV-1 until 1981 when molecular evidence became available which allowed for the differentiation between the two viruses (433). Sequence information, therefore, substantiates the view that EHV-1 and EHV-4 are two closely related but distinct herpesviruses of the horse (434). The detailed pathogenesis of EHV-4 has not been well studied but the infection mirrors that of EHV-1 during the early onset. EHV-4 infection begins with virus replication in mucosal epithelial cells of the upper respiratory tract following inhalation of infectious aerosols or contact with infected fomites. However, the pathogenicity, extent of viral replication and tissue destruction in horses caused by EHV-4 are far lower than those observed for EHV-1 (434). It has been suggested that the biological difference in pathogenicity between EHV-1 and EHV-4 can be attributed to the difference in cellular tropism between the two viruses (5). While primary infection of EHV-4 has been reported to be limited mainly to the epithelial cells of the upper respiratory tract of a susceptible host (435), in vivo replication of EHV-4 in endothelial cells has also
been described (436). It has, however, been demonstrated that EHV-4 has less tropism for mononuclear cells and is less efficient in infecting these cells when compared to EHV-1 (5, 215). Consequently, leukocyte-associated viremia which is a prerequisite for the induction of abortion or neurologic disease in EHV-1 is not a common feature of EHV-4 infection (215).

The type-I IFN response is critical in restricting viral spread from infected to non-infected cells and several viruses have evolved mechanisms to evade this potent antiviral system. The action of EHV-1 on the host cell type-I IFN system during infection has been better studied than that of EHV-4. Recently, we have demonstrated in cell culture that an EHV-1 strain isolated from a neuropathogenic outbreak and possessing the neuropathogenic genotype, T953, has the ability to suppress type-I IFN response at 12 hpi when co-infected with either of the IFN inducers, Sendai virus or Poly-I:C (406). This virus strain was not only successful in downregulating the type-I IFN response but was also capable of inhibiting downstream type-I IFN mediated antiviral activity in vitro (31). Some of the mechanisms determined to be used by this EHV-1 strain to downregulate the type-I IFN response include interference with the nuclear translocation of STAT-1 protein (31) and the disruption of the interferon regulatory factor-3 (IRF-3) signaling pathways (402). Both STAT-1 and IRF-3 are important transcription factors that are indispensable for the sensitization and induction of type-I IFN. Thus it is clear that, in cell culture, the neuropathogenic T953 strain of EHV-1 can overcome the type-I IFN response of the host cell. For this reason, we hypothesized that neuropathogenicity is correlated with the
capacity for a reduced type-I IFN response in the host cell. If true, that would suggest that low type-I IFN induction in the host cell might be a contributing mechanism for neuropathogenicity. To test our hypothesis, here we compared the type-I IFN response between EHV-1 strains possessing the neuropathogenic genotype and those with the non-neuropathogenic genotype using our established cell culture system. We also compared the type-I IFN response following infection with the two closely related but different EHV subtypes: EHV-1 and EHV-4.

2.2 Materials and methods

2.2.1 Cells and viruses

Equine pulmonary artery endothelial cells (EECs; (437)) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc.) with 10% iron-supplemented bovine calf serum (BCS, Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 200 mM L-glutamine (Life Technologies, Carlsbad, CA) in a humidified incubator at 37°C with 5% CO₂. A total of 10 EHV-1 isolates including T953, a well-characterized, neuropathogenic strain also known as the Findlay strain isolated from a nasopharyngeal swab of a horse suffering from an EHV-1 neurologic disease (299), were included in the study. In addition to T953, the other EHV-1 isolates were: AB4, T313, T970, TX06, T75, T186, T220, T262, and T61. An isolate of EHV-4, T445, was also included. To prepare working virus stocks, confluent EECs were infected with EHV-1 at a multiplicity of infection (MOI) of 0.1. After the virus had produced
nearly 100% cytopathic effect (CPE), the tissue culture fluid (TCF) containing the virus was freeze-thawed three times and clarified at 2000 x g for 30 min at 4°C, filtered through 0.45 μm cellulose acetate membrane filters (Thermo Scientific Nunc, Pittsburgh, PA) and purified by ultracentrifugation at 100,000 x g for 4 h at 4°C through a 20% sucrose cushion. The virus pellet was re-suspended in DMEM with 2% BCS, sonicated briefly, aliquoted in 100μl volumes and stored at -80°C until further use. The infectious virus titer was determined by plaque assay in EECs as described (438) with slight modifications. Briefly, confluent EECs propagated in 6-well plates were infected with 10 fold serial dilutions of cell culture supernatant containing virus. For each dilution of the virus, duplicate wells were infected. The virus was let to adsorb for 1 h, at 37°C with 5% CO₂. Unadsorbed viruses were removed by washing and 0.75% carboxymethylcellulose (CMC) media (Sigma-Aldrich, St. Louis, MO) was added to each well and the plates were further incubated in a humidified 5% CO₂ atmosphere at 37°C for 96 h. The CMC media was removed and the infected cells stained with 1% crystal violet (CV) solution in buffered formalin (10%) for plaque visualization. A total of 30 plaques were photographed for each virus strain and the plaque sizes were measured using ImageJ software (439).

Genotyping of field isolates of EHV-1 was carried out as previously described (287) with slight modification. The viruses were grouped into either the neuropathogenic or the non-neuropathogenic genotype of EHV-1 based on the detection of either G₂₂₅₄ or A₂₂₅₄ in the viral DNA polymerase.
2.2.2 

*Viral infections*

EECs were plated into 6-well culture plates (Corning, NY) 48 h prior to infection to obtain more than 90% confluency at the time of infection. Monolayer cells were infected with the various virus strains at an MOI of 5 for 1 h in a humidified incubator at 37°C with 5% CO$_2$. In parallel, cells were either mock-infected with virus diluent (negative control) or treated with 80 µg/ml of Poly-I:C (positive control). The cells were then washed with D-PBS and incubated with complete growth medium for 12 h. All experiments were performed in duplicate and repeated on three independent days.

2.2.3 

*RNA extraction and real-time RT-PCR assay*

Total cellular RNA was extracted using QiaAmp RNeasy plus mini kit (Qiagen Inc. Valencia, CA) from EHV-1-infected and control EECs at 12 hpi according to the manufacturer’s protocol. The quantity and quality of the cellular RNA were examined by OD$_{260}$/OD$_{280}$ measurement using the Synergy H1 hybrid plate reader (Biotek, Winooski, VT). One microgram of total cellular RNA was reverse transcribed as described (440) using 0.5 µg oligo dT primer. Equal amounts of cDNA were used for the transcription analysis of different genes by TaqMan real-time PCR using specific primers and probes in a ViiA™ 7 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The sequences of primers and probes used in this study are listed in Table 2.1. All reactions were performed in duplicate. The RT-PCR data were normalized using the equine ribosomal protein large P0 (RPLP0) transcript as an endogenous control. The PCR efficiency for all reactions was assessed by LinReg software (441). Fold
changes in the gene expression were calculated using the comparative ΔΔCT method for relative quantification (RQ) (442), using the average Ct value of mock-infected samples for each individual gene as the calibrator.

2.2.4 IFN-β reduction score

To quantify the degree of reduction of IFN-β transcription relative to positive control at 12 hpi, each virus was assigned a reduction score based on the algorithmic expression below. Reduction Score = \[\log_{10}(\text{Poly-I:C}) - \log_{10}(\text{Virus})\] / \[\log_{10}(\text{Poly-I:C}) - \log_{10}(\text{Mock})\]. Where each term (Poly-I:C, Virus, Mock) is the measured mean RQ value for IFN-β obtained by RT-PCR. Using this approach, we scored all the EHV-1 isolates based on their ability to induce IFN-β on a scale of 0 to 1. Scores approaching 0 indicate little reduction (IFN expression is similar to that stimulated by Poly-I:C), and scores approaching 1 indicate strong reduction (IFN expression is markedly reduced compared to Poly-I:C).

2.2.5 Type-I interferon bioassay

The inhibition of vesicular stomatitis virus (VSV)-induced cytopathic effect in Madin-Darby bovine kidney (MDBK) cells treated with the cell supernatants harvested at different hpi was measured using the type-I IFN bioassay as previously described (406, 443, 444). Briefly, confluent MDBK cells were treated with two-fold serially diluted supernatant for 24 hrs to stimulate type-I IFN protein secretion in the cell monolayer. Afterwards, the samples were removed and the cells infected with VSV at an MOI of 0.1. Each plate was incubated with virus and cell control wells respectively. Serially diluted recombinant EqIFN-α (Kingfisher
Biotech, Inc., Saint Paul, MN) was used as positive control. After 27 h of incubation, residual cells were fixed and stained with crystal violet for 2 h and the IFN bioactivity was read as an endpoint of a dilution providing 50% protection to cell monolayer expressed as Laboratory Units (LU)/ml.

2.2.6 Statistical methods

Data from the study were analyzed either by Student’s t-test or by ANOVA with pairwise multiple comparison procedures by Tukey’s HSD test using GraphPad Prism version 6.04 (GraphPad Software, Inc., La Jolla, CA). P-Values less than 0.05 except otherwise stated were considered to be statistically significant.

2.3 Results

2.3.1 Identification and genotyping of EHV-1 strains

To ensure that the EHV-1 strains included in the study were properly classified into either the neuropathogenic or the non-neuropathogenic group, we genotyped each virus isolate. This was done using a standardized RT-PCR assay that is able to detect the presence of an A\textsubscript{2254}→G\textsubscript{2254} SNP in the ORF 30 gene of the virus (Equine Diagnostic Solutions, Lexington, KY). The strains possessing the neuropathogenic genotype, having an A\textsubscript{2254}→G\textsubscript{2254} SNP in the ORF 30 gene of the virus, had lower melting temperatures when compared to the strains with A\textsubscript{2254} classified as the non-neuropathogenic genotype (Fig. 2.1.A and 2.1.B).
2.3.2 Effects of EHV-1 strains on IFN-β gene expression following in vitro infection of EECs

All the EHV-1 isolates included in the study, irrespective of their genotype, had reduced IFN-β mRNA expression level at 12 hpi compared to the positive control (Fig. 2.2.). We focused on IFN-β gene expression as the equine genome contains fewer alleles of this gene compared to IFN-α (307). The mean expression of IFN-β mRNA following 12 hpi was then compared between the two genotypes. The neuropathogenic group had a mean log10 RQ value for IFN-β mRNA expression of 0.90 ± 0.11 in EECs, while the non-neuropathogenic group had a mean log10 RQ value of 0.86 ± 0.21. Both the EHV-1 strains possessing the neuropathogenic genotype and the ones possessing the non-neuropathogenic genotype showed low induction of IFN-β mRNA by about the same amount compared to Poly-I:C.

To quantitate the reduction of the type-I IFN response relative to positive control by individual EHV-1 strains following infection, we generated an algorithmic formula as detailed in Materials and Methods. With this, we were able to determine the RQ value of IFN-β mRNA from an EHV-1 infected cell with respect to those from the mock-infected and Poly-I:C-treated cells. We then compared the mean IFN-β reduction scores between the neuropathogenic and the non-neuropathogenic strains of EHV-1. The IFN-β reduction scores of the neuropathogenic strains ranged from 0.58 to 0.84 with a mean of 0.72 ± 0.04 and those of the non-neuropathogenic strains ranged from 0.55 to 0.86 with a mean of 0.74 ± 0.05 (Table 2.2.). Data from our study indicate no statistically significant
difference (p=0.76) in the type-I IFN reduction scores between the neuropathogenic and the non-neuropathogenic groups.

2.3.3 Comparison of the plaques sizes of EHV subtypes (1 and 4)

Since the type-I IFN system plays an important role in restricting viral replication and viral cell-to-cell transmission (445), we compared the plaque sizes produced by the 10 neuropathogenic and the non-neuropathogenic strains of EHV-1 in EECs. With increased type-IFN production following EHV-1 infection, we would expect to see smaller plaques and vice versa. Our finding indicates no statistically significant difference in the sizes of the plaques produced by either group of EHV-1 (P=0.6) as both the neuropathogenic and the non-neuropathogenic genotype produced plaque sizes of approximately 7.2 mm$^2$ and 7.0 mm$^2$ respectively. Additionally, both EHV-1 genotypes produced similar viral titers in EECs (data not shown).

We also compared the sizes of plaques produced by the T953 strain of EHV-1 and the T445 strain of EHV-4 to gain insight into their cell-to-cell spread in EECs. The T953 strain of EHV-1 had a statistically significantly (P=0.0079) larger mean plaque size of about 8.6 mm$^2$ compared to EHV-4, T445 strain with a mean plaque size of 5.30 mm$^2$.

2.3.4 Comparison of IFN-β mRNA expression between EHV-1 (T953 strain) and EHV-4 (T445 strain).

We examined the association between pathogenicity and suppression of type-I IFN response by quantifying IFN-β gene transcription following infection of EECs with the EHV-1 T953 strain and the EHV-4 T445 strain. The pattern of
induction and suppression of IFN-β mRNA following infection with either EHV-1 or EHV-4 is shown in Fig. 2.3. Findings from our study, though not reaching statistical significance, showed a trend indicating that EHV-1 induced more IFN-β mRNA at 3 hpi than EHV-4 (Fig. 2.3.A). The maximum induction of type-I IFN was observed at 6 hpi for EHV-1 infection and this was followed by a decline at 12 hpi. On the contrary, EHV-4 seemed to be a non-potent inducer of IFN-β mRNA at 3 hpi but the IFN response increased steadily at 6 and 12 hpi respectively. At 6 hpi, EHV-1 has induced twice as much IFN-β mRNA compared to EHV-4 in EECs. However, following 12 hpi, EHV-1 has caused a statistically significant (P=0.0087) decline in the IFN-β mRNA, whereas EHV-4 has stimulated increased IFN-β mRNA even further. By 18 hpi, the kinetics of IFN-β mRNA expression in EECs following EHV-1 infection indicated further reduction of IFN-β mRNA while the EHV-4 IFN-β mRNA level was unchanged (data not shown).

We previously showed that EHV-1 suppressed IFN-β responses even in the presence of an outside inducer such as Poly-I:C or Sendai virus (406). To evaluate the direct effect of either EHV-1 or EHV-4 on the induction of IFN-β mRNA stimulated by Poly-I:C treatment, we infected EECs with either EHV-1 or EHV-4 and also treated with Poly-I:C simultaneously. EHV-1 but not EHV-4 infection in the presence of Poly-I:C treatment produced a significant increase in the transcription of IFN-β mRNA at 3 hpi compared to Poly-I:C only positive control (Fig. 2.3.B). However, there was no significant difference in the IFN-β transcription between the Poly-I:C only positive control and either EHV-1 or EHV-
4 infection combined with Poly-I:C treatment at 6 hpi. By 12 hpi, EHV-1 significantly reduced the IFN-β transcription in the presence of Poly-I:C treatment when compared to Poly-I:C only positive control as we have shown previously (Sarkar et al., 2015), whereas EHV-4 infection in the presence of Poly-I:C treatment significantly increased IFN-β mRNA transcription.

2.3.5 Effect of EHV subtypes (1 and 4) on type-I IFN protein activity

To assess whether the IFN-β mRNA expression following infection are reflective of the type-I IFN protein activity, we performed a type-I IFN bioassay. This assay measures the protective effect of total type-I IFN protein in adjacent cells following VSV infection in MDBK cells. At 3 hpi (Fig. 2.4.A.), both EHV-1 and EHV-4 produced less type-I IFN protein activity when compared to the positive control (Poly-I:C or Sendai Virus) with EHV-4 inducing the least IFN of all, although the differences were not statistically significant (P= 0.24). Following 6 hpi (Fig. 2.4.B.), the most striking observation to emerge from the data comparison was that EHV-1 suppressed the type-I IFN protein activity induced by Sendai virus (P=0.03). Remarkably, by 12 hpi (Fig. 2.4.C.), EHV-1 had significantly suppressed the type-I IFN protein activity produced by Poly-I:C and Sendai virus respectively, whereas EHV-4 significantly enhanced type-I IFN protein activity produced by Poly-I:C. These results offer support for our IFN-β mRNA data, with EHV-1 but not EHV-4 capable of suppressing type-I IFN response by 12 hpi. Interestingly, while EHV-1 failed to induce any type-I IFN protein activity at 12 hpi, EHV-4 had induced more type-I IFN protein by 12 hpi.
2.4 Discussion

For a virus to be successful, it must possess factors that allow it to evade the key components of the host defensive structure: the physical and chemical barriers, the innate immunity and the adaptive immunity. EHV-1, like other herpesviruses, possesses an array of impressive immune evasive strategies (293, 446, 447) that are critical for the successful establishment of disease process once the virus perpetuates in the host cell. Here, we tested a hypothesis that neuropathogenicity is correlated to the host type-I IFN response. We also compared the suppression of type-I IFN between the EHV-1 T953 strain and a strain of EHV-4, T445, to further understand how widespread the IFN suppressive function is found among equine alpha herpesviruses.

Firstly, we screened a panel of viruses of both the neuropathogenic and the non-neuropathogenic genotypes for their effect on host cell type-I IFN response. There was no statistical difference in the level of expression of IFN-β mRNA between the neuropathogenic and the non-neuropathogenic groups. This finding suggests that the neuropathogenic strains of EHV-1 do not cause a lower type-I IFN induction than the non-neuropathogenic group, refuting our hypothesis that a reduced IFN response may contribute to the neuropathogenic phenotype. A lower induction of type-I interferon appears to be an immune evasion strategy used commonly by all EHV-1 strains. It is apparent that both the G_{2254} and the A_{2254} genotypes of EHV-1 possess viral genes employed to escape the host innate immune response during infection. Like EHV-1, other herpesviruses including HSV, bovine herpesvirus, varicella-zoster virus, and mouse...
cytomegalovirus, first induce and then inhibit IFN-α/β production (315, 448-451). For example, alpha herpesviruses like BoHV-1 and HSV-1 express an immediate early (IE) protein, infected cell protein 0 (ICP0), in the infected cells, which interferes with multiple steps of the type-I IFN response. EHV-1 also possess a homolog of this viral protein, EICP0, but its role with regards to interference with type-I IFN response in the equine host has not been documented. Further studies are, therefore, required to illuminate whether EICP0 exhibit similar roles on host type-I IFN response as observed in both BoHV-1 and HSV-1 ICP0.

To confirm similarity of behavior between the 2 genotypes, we then evaluated the ability of both EHV-1 genotypes to produce visible plaques in EECs. Our results indicate no difference in the plaque sizes between the neuropathogenic and the non-neuropathogenic strains. Despite the presence of a non-synonymous mutation (A2254 → G2254) in the DNA polymerase of the neuropathogenic group of EHV-1, which has been shown to affect virus replication based on titer (411), the plaque size remained unaltered in EECs. Our finding agrees with that of Goodman and co-workers who reported no difference in the replication properties of both genotypes of EHV-1 in equine fibroblasts in vitro (452). However, the G2254 genotype appears to play a key role in determining the level of viremia and endothelial cell infection in horses in vivo (5, 452-454). A possible explanation for this difference compared to our study could be that in natural infection, viruses of the G2254 genotype due to their better replication efficiency (272), are effectively transmitted to the vasculature of pregnant uterus and CNS highlighting the limitation of our in vitro system. In the
future, we hope to extend our research along those lines in a different model of immediate relevance for the initial stage of EHV-1 pathogenicity.

Both EHV-1 and EHV-4 are successful viral pathogens of horses and their ability to inflict disease is influenced by their possession of unique biological features. Findings from our study indicate that the two virus types have a different pathogenic potential for stimulating and downregulating type-I IFN response. While EHV-1 was more competent both in inducing and shutting down the type-I IFN response of the host cell even in the presence of Poly-I:C or SeV, EHV-4 only weakly induced type-I IFN over a period of time without downregulating it. Our data suggest that the suppression of type-I IFN activity by EHV-1 may promote viral cell-to-cell spread during disease process more ably than EHV-4. This is corroborated by finding from another study (215) which reported a significantly increased plaque size in respiratory epithelial cells (RECs) infected with EHV-1 compared with EHV-4 at different time points, indicating a reduced lateral spread of EHV-4 compared to EHV-1 in vitro. Since EECs (equine endothelial cells) are specialized epithelial cells lining blood vessels, it is not inconceivable that different results could arise if our plaque assay had made use of a totally different cell line. While we have the complete sequence information of T953, we do not have the sequence of T445 used in this study. It will be interesting to compare the homology of both viruses to reveal the extent of similarity between both EHV subtypes. Taken together, there is evidence to suggest that the T953 strain of EHV-1 possess the gene(s)
responsible for suppression of host type-I IFN response while EHV-4, T445 strain, either lacks the gene or the gene is silenced.

Type-I IFN plays an important role in triggering the transcription of a diverse set of genes known as IFN-stimulated genes (ISGs) that create a global antiviral environment within the host cell. A low induction of type-I IFN response by both genotypes of EHV-1 will prevent the production and further release of antiviral proteins (31), thus facilitating viral spread from infected to non-infected cells. Most viruses equipped with the ability to down-regulate the type-I IFN response use non-structural (NS) viral proteins for that purpose, which are otherwise non-essential for virus growth. For example, the US11 protein of HSV-1 binds to retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated protein 5 (MDA-5) in order to inhibit their downstream signaling pathways, thereby preventing the production of IFN-β (395). For this reason, it is plausible that the viral factor responsible for the low induction of host IFN-β by EHV-1 is a non-structural protein other than the viral DNA polymerase. The putative EHV-1 gene product that is involved in the reduction of host IFN-β still remains to be identified. However, our study has established that both the neuropathogenic and non-neuropathogenic genotypes of EHV-1 are equally competent to provoke a low induction of type-I IFN response.

2.5 Conclusion

In this study, we provide evidence that the ability to evade a key component of the host innate immunity is not a unique feature of the neuropathogenic genotype of EHV-1 as both genotypes of EHV-1 were equally capable of
reducing IFN-β expression to low levels \textit{in vitro}, which could contribute to viral spread to distant sites for disease manifestation. Further, while EHV-1 induced a type-I IFN response early during infection, it also rapidly reduced the IFN activity at late time points, unlike EHV-4 that weakly induced type-I IFN without suppressing it. Future studies should focus on identifying the key viral proteins that exert these effects on the type-I IFN response by EHV-1.
Tables.

Table 2. 1 Primers and probes for RT-PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequences (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>EqRPLP0</td>
<td>Fwd: CTGATTACACCTCTCCCACTTGCT</td>
</tr>
<tr>
<td></td>
<td>Rev: AGCCACAATGCAGATGGATCA</td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-AAGCCTTGACCTTTTC-NFQ</td>
</tr>
<tr>
<td>EqIFN-β</td>
<td>Fwd: AATGGCCTCTCTGCTGT</td>
</tr>
<tr>
<td></td>
<td>Rev: CCGAAGCAAGTCATAGTCACACA</td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-CTCCACCACGGCTC-NFQ</td>
</tr>
</tbody>
</table>

Table 2. 2 IFN-β reduction scores following EHV-1 infection.

<table>
<thead>
<tr>
<th>EHV-1 genotype</th>
<th>EHV-1 strain</th>
<th>IFN-β reduction score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropathogenic</td>
<td>T953</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>AB4</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>T313</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>T970</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>TX06</td>
<td>0.58</td>
</tr>
<tr>
<td>Non-neuropathogenic</td>
<td>T262</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>T75</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>T186</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>T220</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>T61</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Interferon reduction scores were calculated according to the equation: 
\[\frac{\log_{10} (\text{Poly-I: C}) - \log_{10} (\text{Virus})}{\log_{10} (\text{Poly-I: C}) - \log_{10} (\text{Mock})}\].
Figures.

Figure 2.1 Melting curves of EHV-1 isolates. (A). Viruses were screened for the presence or absence of an SNP in their DNA polymerase encoded by ORF 30 using a real-time PCR assay. (B). As controls, a neuropathogenic and a non-neuropathogenic plasmid of EHV-1 were included in the assay.
Expression levels of IFN-β mRNA following EHV-1 infection. EECs were either infected with the indicated EHV-1 strain at an MOI of 5, or mock-infected (as represented by M) or Poly-I:C-treated (as represented by P). At 12 hpi, the cells were lysed and equine IFN-β mRNA was quantified by real-time PCR as described in Materials and Methods. Data were normalized to levels of the endogenous control equine RPLPO mRNA at the same time point. Each bar represents the mean and standard deviation from three independent experiments.

IFN-β mRNA expression levels following infection with EHV-1 and EHV-4. (A) EECs were either infected with the T953 strain of EHV-1 (E1) or with the T445 strain of EHV-4 (E4). As controls, cells were either mock-infected or treated with 80 µg/ml of Poly-I:C. After 3, 6 and 12 hpi, cells were lysed and equine IFN-β mRNA was quantified by real-time PCR. The expression levels of IFN-β mRNA were then compared between T953 and T445. (B) EECs were either stimulated with Poly-I:C (80 µg/ml) or both stimulated with Poly-I:C and infected with either EHV-1 T953 or EHV-4 T445 together for 3 h, 6 h, and 12 h. IFN-β mRNA was quantified as described. Each bar represents the mean and standard deviation from three independent experiments. Differences were significant at p<0.0001 (****) or p<0.01 (**) while 'ns' denotes non-significant differences.
Figure 2.4  Effect of EHV-1 and EHV-4 infection on levels of type-I IFN biological activity. EECs were mock infected (M) or infected with T953 only (E1) at an MOI of 5 or infected with T445 only (E4) at an MOI of 5 or co-infected with T953 and Sendai Virus (SeV+E1), or co-incubated with T953 and Poly-I:C (P+E1) or co-infected with T445 strain and Sendai Virus (SeV+E4), or co-incubated with T445 and Poly-I:C (P+E4). As positive controls, EECs were either infected with Sendai virus (SeV) at an MOI of 5 or treated with 80 µg/ml of Poly-I:C (P). At indicated times post-infection; (A) 3 hpi; (B) 6 hpi; and (C) 12 hpi, harvested cell supernatants were tested for their type-I IFN bioactivity. Each bar represents the mean and standard deviation from three independent experiments. Differences were significant at p<0.0001 (**), p<0.01 (**), and p<0.05 (*) while 'ns' denotes non-significant differences.
CHAPTER 3: EQUINE HERPESVIRUS TYPE 1 TARGETS THE SENSITIZATION AND INDUCTION STEPS TO INHIBIT TYPE-I INTERFERON RESPONSE IN EQUINE ENDOTHELIAL CELLS.

Abstract

Equine herpesvirus type 1 (EHV-1) is a viral pathogen of horse populations worldwide spread by the respiratory route and is known for causing outbreaks of neurologic syndromes and abortion storms. Previously, we demonstrated that an EHV-1 strain of the neuropathogenic genotype, T953, downregulates the IFN-β response in vitro in equine endothelial cells (EECs) at 12 h post-infection (hpi). In the current study, we explored the molecular correlates of this inhibition as clues towards an understanding of the mechanism. Data from our study revealed that EHV-1 infection of EECs significantly reduced both TLR3 and TLR4 mRNA expression at 6 hpi and 12 hpi. While EHV-1 was able to significantly reduce IRF9 mRNA at both 6 hpi and 12 hpi, the virus significantly reduced IRF7 mRNA only at 12 hpi. EHV-1 did not alter the cellular level of JAK1 at any time point. However, EHV-1 reduced the cellular level of expression of TYK2 at 12 hpi. Downstream of the JAK1-TYK2 signaling EHV-1 blocked the phosphorylation and activation of STAT2 when co-incubated with exogenous IFN, at 12 hpi, although not at 3 or 6 hpi. Immunofluorescence staining revealed that the virus prevented the nuclear translocation of STAT2 molecules confirming the virus-mediated inhibition of STAT2 activation. The pattern of suppression of phosphorylation of STAT2 by EHV-1 implicated viral late gene expression. These data help illuminate how EHV-1 strategically inhibits the host innate immune defense by limiting steps required for type-I IFN sensitization and induction.

KEYWORDS: EHV-1, Type-I IFN, TLR, STAT2, TYK2, ISGF3, Innate immunity.
3.1 Introduction

Equine herpesvirus type 1 (EHV-1) is a large DNA virus belonging to the genus *Varicellovirus* of the *Herpesviridae* family (76). The virion structure, size and replicative strategy of EHV-1 is similar to those of other herpesviruses such as human herpes simplex virus, varicella-zoster virus, and bovine herpesvirus-1 (82). The virus is enzootic in the world’s horse population predisposing horses to high risk of infection. Most horses acquire the infection at a young age and become latent carriers throughout their life (5, 10), with recrudescence into active infection when the animals are under stress (10, 77). EHV-1 produces a constellation of disease syndromes including upper respiratory tract infection, early neonatal death in foals, sporadic or epizootic abortions in pregnant mares, and a devastating form of neurologic disease called equine herpesviral myeloencephalopathy (EHM) in adult horses that is fatal in 20-50% of cases (455-457). EHM has been associated with an A\textsuperscript{2254}\textrightharpoonupG\textsuperscript{2254} mutation in the viral DNA polymerase (ORF30). Generally, neuropathogenic strains such as the T953 strain used here have aspartic acid at position 752 whereas non-neuropathogenic strains possess asparagine (268, 269). In field outbreaks, this association is strong but not absolute, and there may be other factors that could contribute to neuropathogenicity (109, 270).

Upon initial viral insult, many host cells rely on the non-specific effects of biological regulatory proteins called IFN to contain the viral spread and prevent infection of bystander cells (27). The induction of type-I IFN response following viral infection occurs in 3 phases- sensitization, induction, and amplification
In the initial sensitization phase, viral motifs or pathogen associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) present in the cells to initiate antiviral signal transduction, featuring coordinated activation of transcription factors including interferon regulatory factor 3 (IRF3), IRF7, and nuclear factor-κB (NF-κB) which induce IFN-β at a very low level (329). In the context of a virus infection, TLR3, TLR4, and TLR9 are important for the signaling that initiates type-I IFN production. TLR3 recognizes double-stranded RNA (dsRNA), an intermediate of most DNA viruses during replication (458), while TLR4 and TLR9 recognize viral glycoproteins and CpG DNA respectively (459, 460). Both TLR3 and TLR4 signal through activation of IRF3, which then dimerizes, translocates into the nucleus, binds to the promoter of IFN-β, and induces its transcription (461, 462). On the other hand, TLR9 signals through the activation of IRF7 whose subsequent nuclear translocation upon homodimerization results in upregulated type-I IFN genes (463). In the succeeding induction phase, secreted IFN-β binds to its cognate receptors present ubiquitously on cell surfaces, inducing phosphorylation-activation of receptor-associated Janus-activated kinases (JAKs) including tyrosine kinase 2 (TYK2) (21). Activated JAK1 and TYK2 phosphorylate signal transducer and activator of transcription-1 (STAT1) and STAT2 which bind to IRF9 forming the interferon-stimulated gene factor 3 (ISGF3) heterocomplex (326). ISGF3 translocates into the nucleus and binds to the IFN-stimulated response elements (ISRE) of different IFN inducible genes including IRF7 which enhances their transcription (464-466). Activated IRF3
forms a heterodimer with IRF7, translocates into the nucleus, and binds to the promoter of IFN-α genes and stimulates their transcription (329, 462). In the amplification phase, the synergistic effect of IFN-α and IFN-β induces enhanced transcription of a diverse set of genes known as IFN-stimulated genes (ISGs) which create a global antiviral environment (467, 468).

The impact of EHV-1 on host IFN responses has not been exhaustively studied. Previous studies from our group have revealed that in vitro in EECs, neuropathogenic T953 virus infection is capable of inhibiting host type-I IFN by 12 hpi and it does so by blocking certain transcriptional factors required for type-I IFN response (31, 402, 406). More recently, we showed that EHV-1 strains with the neuropathogenic genotype as well as those with the non-neuropathogenic genotype are both able to inhibit type-I IFN production in EECs thereby enabling viral spread to neighboring cells (469). By suppressing the host type-I IFN response, we predict that T953 interferes with one or more transcription factors required for type-I IFN production. To test this hypothesis, here we investigated the impact of T953 on key molecules required for type-I IFN sensing and induction. We also investigated the involvement of viral gene expression on the type-I IFN pathway to gain insight on whether a productive viral replication cycle is required for type-I IFN inhibition.

3.2 Materials and methods

3.2.1 Cells and viruses

Equine pulmonary artery endothelial cells (EECs; (437)) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc.) with 10% iron-
supplemented bovine calf serum (BCS, Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 200 mM L-glutamine (Life Technologies, Carlsbad, CA) in a humidified incubator at 37 °C with 5% CO₂. A well characterized EHV-1 strain T953 isolated from a case of equine herpesvirus myeloencephalopathy (EHM) at the Veterinary Medical Teaching Hospital, University of Findlay, Ohio in 2003 (299) was used for the study. To prepare working virus stocks, confluent EECs were infected with the virus at a multiplicity of infection (MOI) of 0.1. After the virus had produced nearly 100% cytopathic effect (CPE), the tissue culture fluid (TCF) containing the virus was freeze-thawed three times and clarified at 2000 x g for 30 min at 4°C, filtered through 0.45 μm cellulose acetate membrane filters (Thermo Scientific Nunc, Pittsburgh, PA), and purified by ultracentrifugation at 100,000 x g for 4 h at 4°C through a 20% sucrose cushion. The virus pellet was re-suspended in DMEM with 2% BCS, sonicated briefly, aliquoted in 100µl volumes and stored at -80°C until further use. The infectious virus titer was determined by plaque assay in EECs as described (438) with slight modifications. Briefly, confluent EECs propagated in 6-well plates were infected with 10 fold serial dilutions of cell culture supernatant containing virus. For each dilution of the virus, duplicate wells were infected. The virus was adsorbed for 1 h, at 37°C with 5% CO₂. Unadsorbed viruses were removed by washing and 0.75% carboxymethylcellulose (CMC) media (Sigma-Aldrich, St. Louis, MO) was added to each well and the plates were further incubated in a humidified 5% CO₂ atmosphere at 37°C for 96 h. The CMC media
were removed and the infected cells stained with 1% crystal violet (CV) solution in buffered formalin (10%) for plaque visualization.

3.2.2 Viral infections

EECs were plated into 6-well culture plates (Corning, NY) 48 h prior to infection to ensure more than 90% confluency at the time of infection. Monolayer cells were infected with EHV-1, T953 strain, at an MOI of 5 for 1 h in a humidified incubator at 37 °C with 5% CO₂. In parallel, cells were either mock-infected with virus diluent (negative control) or treated with 80 μg/ml of Poly-I:C (positive control) unless otherwise stated. The cells were then washed with D-PBS and incubated with complete growth medium for 3 h, 6 h, and 12 h respectively. All experiments were performed in duplicate and repeated on three independent days.

3.2.3 RNA extraction and real-time RT-PCR assay

Total cellular RNA was extracted using QiaAmp RNeasy plus mini kit (Qiagen Inc. Valencia, CA) from EHV-1-infected and controls’ EECs at 3 h, 6h, and 12 h according to the manufacturer’s protocol. The quantity and quality of the cellular RNA were determined by OD₂₆₀/OD₂₈₀ measurement using the Synergy H1 hybrid plate reader (Biotek, Winooski, VT). One μg of total cellular RNA was reverse transcribed as described (440) using 0.5 μg oligo dT primer. Equal amounts of cDNA were used for the transcription analysis of different genes by TaqMan real-time PCR using inventoried primers and probes (Thermo Scientific, Pittsburgh, PA) in a Viia™ 7 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Details of the primers and probes used in this study are
provided in Table 1. All reactions were performed in duplicate. The RT-PCR data were normalized using the equine ribosomal protein large P0 (RPLP0) transcript as an endogenous control. The PCR efficiency for all reactions was assessed by LinReg software (441). Fold changes in the gene expression were calculated using the comparative ΔΔCT method for relative quantification (RQ) (442), using the average Ct value of mock-infected samples for each individual gene as the calibrator.

3.2.4 Western immunoblotting assay

EECs were infected with EHV-1, T953 strain, at an MOI of 5. At 30 minutes prior to the end of experiment, infected cells were treated with rEqIFN-α at 1000 IU/ml following established protocols (31, 470, 471). Cells were then washed in cold PBS and solubilized in RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX) enriched with phosphatase inhibitor (Thermo Scientific, Pittsburgh, PA) on ice. The protein concentration was measured using BCA protein assay kit (Thermo Scientific, Pittsburgh, PA) and measured using the Synergy H1 hybrid plate reader (Biotek, Winooski, VT). Equal amount of protein were then separated in 10% sodium dodecyl polyacrylamide resolving gels and transferred onto PVDF membranes (BioRad, Hercules, CA). Following 1 h of blocking, membranes were incubated with the respective primary antibody (anti-JAK1, anti-TYK2, anti-phospho-STAT1, anti-phospho-STAT2, anti-β-actin, and anti-lamin A/C all used at 1:1000 dilution) in 5% bovine serum albumin at 4°C overnight. The membranes were then washed and incubated with corresponding secondary antibody for 1 h at room temperature (RT). Afterwards,
membranes were developed with the aid of an enhanced chemiluminescent substrate (Thermo Scientific, Pittsburgh, PA) and imaged immediately using Azure c600 (Azure Biosystems, Dublin, CA). The relative intensities of expression levels of the respective protein bands were quantified by densitometric analysis using ImageJ software (National Institute of Health, USA).

3.2.5 Cytoplasmic and nuclear protein extraction

To generate subcellular fractionation, cytoplasmic and nuclear proteins were extracted using Thermo Scientific NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's protocol. Briefly, an ice-cold cytoplasmic extraction reagent I (CER I) was added to pelleted cells, vigorously vortexed, and incubated on ice for 10 min. This was followed by the addition of ice-cold cytoplasmic extraction reagent II (CER II). The solution was vortexed, incubated on ice for 1 min and centrifuged at 16,000 x g for 5 min. The supernatant containing the cytoplasmic protein extracts were stored at -80º C while pellets containing the nuclear proteins were re-suspended in ice-cold nuclear extraction reagent, vortexed every 10 min during a total of 40 min incubation on ice. The lysate was centrifuged at 16,000 x g for 10 min and the supernatant containing the nuclear proteins was immediately collected and stored at -80º C. Equal amounts of cytoplasmic and nuclear proteins were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and blotted with antibodies as described.
3.2.6 *Indirect immunofluorescence assay*

Confluent EECs seeded on coverslips (Electron Microscopy Sciences, Hatfield, PA) in 24 well cell culture plates were infected with EHV-1, T953 strain, at an MOI of 3. To minimize rounding-up and clumping of EHV-1-infected cells at an MOI of 5 which may affect the quality of IF images, we decided to test a different infection condition using a lower MOI. At 30 minutes prior to fixation, both infected and uninfected cells were treated with rEqIFN-α at 1000 IU/ml (positive control) or equal volume of media (negative control). The cells were then fixed with 4% paraformaldehyde (PFA) at RT for 30 min and stained as described (31, 472, 473). After 3 washes with 10mM glycine-PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS (Sigma, St. Louis, MO) for 10 min at RT and blocked with 5% normal goat serum in PBS for 30 min at RT. The cells were subsequently incubated with the primary antibodies (rabbit anti-STAT2, 1:100 dilution; and mouse anti-EHV-1 gC, 1:200 dilution) at RT for 1 h. The unbound antibodies were washed off with 10mM glycine-PBS and the cells were incubated with the corresponding secondary antibody conjugated with either Alexa Fluor 488 at 1:200 dilution or Texas Red at 2 µg/ml dilution (Thermo Scientific, Pittsburgh, PA) for 1 h at RT. Finally, the cells were washed and mounted in VECTASHIELD Mounting Medium containing 4’, 6’- diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and observed at X 40 objective lens under an inverted fluorescence microscope (Nikon ECLIPSE Ti, Melville, NY). Images were captured using DS-QiMc-U3 camera, and nuclear STAT2 translocation was quantified using Nikon NIS-Elements software following
background subtraction and thresholding for each channel. The STAT2 fluorescence was measured without prior knowledge of cells expressing EHV-1 gC.

3.2.7 Antibodies and other reagents

Rabbit anti-STAT1 and rabbit anti-STAT2 primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti-β-actin primary antibody, rabbit anti-JAK1, rabbit anti-TYK2, rabbit anti-phospho-STAT1 primary antibody, Lamin A/C, MEK1/2, and goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, CA). Rabbit anti-phospho-STAT2 primary antibody was purchased from Rockland Immunochemicals Inc. (Limerick, PA). Donkey anti-goat IgG (H+L) secondary antibody, Texas Red, Goat anti-Rabbit IgG (H+L) secondary antibody, Alexa Fluor 488, and phosphonoacetic acid were purchased from Thermo Scientific (Pittsburgh, PA). Poly-I: C and LPS-RB ultrapure were purchased from Invivogen (San Diego, CA). Both the EHV-1 anti-IE (474, 475) and EHV-1 anti-gD (476) primary antibodies were kindly provided by Dr. Dennis O’Callaghan (Louisiana State University). The EHV-1 gC specific primary antibody has been described elsewhere (477) while rEqlIFN-α was purchased from Kingfisher Biotech, Inc. (Saint Paul, MN).

3.2.8 Statistical methods

Data from the study were analyzed by ANOVA with pairwise multiple comparison procedures by Tukey’s HSD test using GraphPad Prism version 6.04 (GraphPad Software, Inc., La Jolla, CA) yielding a multiplicity adjusted P-value
for each comparison. P-Values less than 0.05 except otherwise stated were considered to be statistically significant.

3.3 Results

3.3.1 EHV-1 modulation of TLR3 and TLR4 transcription

In order to assess the impact of T953 on selected pattern recognition receptors, we quantified TLR3 and TLR4 mRNA following infection in EECs. Findings from our study revealed that following 3 hpi, T953 induced about the same amount of TLR3 mRNA as in mock-infected cells and showed no suppressive effect on TLR3 mRNA when co-incubated with the IFN inducer, polyinosinic acid: polycytidylic acid (Poly-I:C) (Fig. 3.1.A). At 6 hpi, the kinetics of induction of TLR3 mRNA were not statistically significantly different (P = 1.0) between EHV-1- and mock-infected cells, but T953 exerted a statistically significant (P < 0.0001) suppressive effect on TLR3 mRNA when co-incubated with Poly-I:C at this time-point. At 12 hpi, T953 infection did not induce any TLR3 mRNA and the virus infection in the presence of Poly-I:C statistically significantly (P < 0.0001) down-regulated TLR3 mRNA by about 54 fold compared to the positive control (Poly-I:C). The pattern of induction of TLR4 revealed no statistically significant (P = 0.76) difference between the treatment groups at 3 hpi (Fig. 3.1.B). At 6 hpi, the virus had induced about the same amount of TLR4 mRNA when compared with mock-infected cells. In the presence of a TLR4 agonist, lipopolysaccharide (LPS), T953 infection had a statistically significant (P = 0.0016), suppressive effect on TLR4 mRNA when compared to the positive control at this time-point. At 12 hpi, there was no difference in the induction of
TLR4 mRNA between mock-infected and T953-infected cells, but the virus in the presence of LPS suppressed TLR4 mRNA by about 27 fold compared to the positive control (LPS). To demonstrate the specificity of EHV-1-mediated downmodulation of TLR3 and TLR4, we evaluated the impact of EHV-1 on equine β-glucuronidase using RT-PCR. Our data revealed that at all studied time-points, EHV-1 did not alter the expression of this cellular gene (data not shown).

**3.3.2 EHV-1 suppression of IRF7 and IRF9 mRNA transcription**

The IRF7 transcription factor plays an important role in regulating the transcriptional activation of virus inducible cellular genes like IFN-α. On the other hand, IRF9 forms a complex termed ISGF3 with activated STAT1 and STAT2 proteins to drive the induction of ISGs following viral infection. To study the impact of T953 on IRF7 and IRF9 transcription factors, we evaluated IRF7 and IRF9 mRNA in EECs following *in vitro* infection using RT-PCR. The kinetics of transcription of IRF7 mRNA following infection with T953 was similar to that of the mock-infected cells at 3hpi and 6 hpi (Fig. 3.2.A). At these time points, the expression of IRF7 mRNA induced by Poly-I:C was unchanged by T953 infection. However, at 12 hpi, T953 infection statistically significantly (P < 0.0001) suppressed IRF7 mRNA transcription induced by Poly-I:C. Similarly, T953 infection had no statistically significant (P = 0.44) effect on the IRF9 mRNA transcription induced by Poly-I:C at 3 hpi (Fig. 3.2.B). However, at 6 hpi, T953 infection caused a statistically significant (P < 0.0001) reduction of IRF9 mRNA in the presence of Poly-I:C. At 12 hpi, the kinetics of transcription of IRF9 mRNA
revealed that T953 had statistically significantly (P < 0.0001) suppressed IRF9 mRNA by about 40 fold compared to levels with Poly-I:C alone.

3.3.3 EHV-1 degrades cellular levels of TYK2 but not JAK1 during infection

Upstream of the signaling cascades leading to type-I IFN production are the JAK1 and TYK2 proteins which play key roles in the phosphorylation and activation of STAT1 and STAT2. To evaluate whether the inhibitory effects of T953 on the type-I IFN response is reflective of the virus’ ability to disrupt these upstream signaling events, we quantitated the cellular levels of JAK1 and TYK2 following T953 infection and also in response to exogenous recombinant equine interferon-α (rEqIFN-α) treatment following infection. Western blot analysis revealed that T953 had no downregulating effect on TYK2 at 3 hpi (Fig. 3.3.A) and 6 hpi (Fig. 3.3.B) either with or without IFN treatment. However, at 12 hpi, the cellular abundance of TYK2 was reduced either in cells infected with T953 alone or in cells infected with T953 and co-incubated with exogenous IFN-α (Fig. 3.3.C). Densitometric analysis revealed that while T953 failed to alter the cellular levels of JAK1 at all the time-points in the study (Fig. 3.3.D), the virus demonstrated an ability to inhibit TYK2 protein (P = 0.0006) in the presence of IFN treatment at 12 hpi (Fig. 3.3.E). These data suggested the possibility of a consequent downstream inhibition of IFN-induced STAT2 phosphorylation.

3.3.4 EHV-1 alters the cellular levels of phosphorylated STAT1 and STAT2 during infection

Both JAKs and STATs are frequently targeted by virally encoded antagonists to inhibit their activation (478). Given the role of ISGF3 in the
downstream signaling events leading to type-I IFN induction, we hypothesized that T953 is capable of blocking the formation of ISGF3 complex in vitro over the course of infection. Here we evaluated the phosphorylation of both STAT1 and STAT2 by Western immunoblotting assay after infection of EECs with T953 in the presence or absence of rEqIFN-α. We previously showed that T953 infection interferes with STAT1 phosphorylation in EECs at 12 hpi (31). We confirmed this result (Fig. 3.4.A) and also found that STAT2 is also inhibited (Fig. 3.4.B-D). Data revealed that T953 infection did not prevent exogenous IFN-induced STAT2 phosphorylation at either 3 hpi (Fig. 3.4.B) or 6 hpi (Fig. 3.4.C) but the virus infection reduced IFN-induced STAT2 phosphorylation in EECs at 12 hpi (Fig. 3.4.D). In the absence of an exogenous IFN stimulation, T953 infection triggered only a small amount of STAT2 phosphorylation in EECs at 6 (Fig. 3.4.C) and 12 hpi (Fig. 3.4.D). The suppressive effect of T953 on both STAT1 and STAT2 phosphorylation when co-incubated with exogenous IFN at 12 hpi did not alter the endogenous abundance of either total STAT1 or total STAT2 molecule. This finding was corroborated by the densitometric analysis of the phosphorylated STAT relative to total STAT proteins. The analysis showed that T953 at 12 hpi only, statistically significantly (P < 0.0001) reduced the phosphorylation of STAT1 and STAT2 proteins when co-incubated with exogenous IFN (Fig. 3.4.E and 3.4.F).

3.3.5 EHV-1 blocks nuclear translocation of STAT2 during infection

The above experiments showed that T953 inhibited the phosphorylation of STAT1 and STAT2 at 12 hpi in the presence of IFN-α treatment. We further
investigated the anti-STAT effect of T953 by using indirect immunofluorescence imaging. Our previous publication showed that T953 blocked nuclear translocation of STAT1 even in the presence of exogenous IFN (31). We hypothesized that T953 also blocks the activation of STAT2, which would be evidenced by cytosolic localization of STAT2 in virus-infected cells and also in virus-infected cells co-incubated with exogenous IFN. By using immunofluorescence, as expected we found an exclusively cytoplasmic retention of STAT2 molecules in a majority of uninfected cells when stained with anti-STAT2 antibody (Fig. 3.5.A). Treatment of the uninfected cells with exogenous IFN-α resulted in redistribution STAT2 molecules leading to nuclear accumulation of these molecules in a majority of cells as expected (Fig. 3.5.B). In contrast, a majority of EHV-1 infected cells exhibited cytoplasmic retention of STAT2 both in the absence (Fig. 3.5.C) and presence (Fig. 3.5.D) of exogenous IFN-α indicating that STAT2 molecules were not efficiently activated. Moreover, nuclear STAT2 quantification of IF images revealed that even in the presence of exogenous IFN-α treatment, EHV-1 infection of EECs produced a statistically significant reduction of nuclear STAT2 when compared to uninfected cells treated with exogenous IFN-α (Fig. 3.5.E). To confirm these findings, we carried out subcellular fractionation following infection or treatment with exogenous IFN-α and quantitated the level of STAT2 molecules in the nuclear and cytoplasmic compartments by Western immunoblotting. Consistent with our immunofluorescence data, T953 reduced the nuclear accumulation of phosphorylated and total STAT2 (Fig. 3.5.F) in infected cells when co-incubated
with exogenous IFN-α. This result provides a plausible mechanistic insight into how T953 blocks the type-I IFN signaling cascade when stimulated by exogenous IFN-α.

3.3.6 Suppression of type-I IFN signaling molecules is mediated by an active EHV-1 gene

We previously showed that active viral replication is necessary for STAT1 interference by T953 (31), and show above that the virus also inhibited STAT2 phosphorylation and subsequent activation. In the above experiments, the downregulation of these signal-transduction molecules in the IFN induction pathway by T953 was demonstrable by 12 hpi. For this reason, we tested the hypothesis that these suppressive effects required active virus gene expression during T953 replication in EECs. To evaluate whether a productive viral infection in EEC is essential for the above effects, we performed similar experiments using UV-inactivated T953 virus. We UV-inactivated T953 by irradiating the virus with $7.2 \times 10^4$ J of 254 nm UV from a distance of 10 cm using a UV Stratalinker 800 (Stratagene, La Jolla, CA). As a control to examine whether this level of UV-inactivation of T953 inactivated virion proteins as well as DNA-directed transcription, we evaluated for T953 gD before and after UV-inactivation. We found that gD of UV-inactivated T953 was still recognized by a gD-specific antibody suggesting that gD likely retained activity and the virus can enter infected EECs (data not shown). The inactivation of T953 was demonstrated by absence of cytopathic effect when used to infect RK-13 cells following 24 h of incubation. EECs were then infected with UV-inactivated T953 at an equivalent
dilution to the infectious T953, and both cellular RNA for RT-PCR and protein for Western immunoblotting were subsequently harvested. Mock infected cells stimulated with either 80 μg/ml of Poly-I:C or 1000 IU/ml of rEqIFN-α were included as positive controls. Results showed that by comparison with Figures 1 and 2, UV-inactivated T953 was unable to statistically significantly downregulate TLR3 and TLR4 mRNA at 3, 6, and 12 hpi compared with the positive control (Fig. 3.6.A and 3.6.B). Similarly, UV-inactivated T953 failed to inhibit IRF7 and IRF9 mRNA at 3, 6, and 12 hpi when compared with the positive control (Fig. 3.6.C and 3.6.D). UV-inactivated T953 also failed to suppress STAT2 phosphorylation (Fig. 3.6.E) when co-incubated with rEqIFN-α at 12 hpi. Since these phenotypes of UV-inactivated T953 contradicted those of the infectious virus, we conclude that an active viral replication was required for suppression of key molecules of type-I IFN signaling by T953 including TLR3/4, and IRF7/9.

3.3.7 A late viral gene of EHV-1 blocks STAT2 activation

Like other herpesviruses, the EHV-1 transcriptome is temporally expressed as immediate early (IE), early (E), and late genes (L) in that sequence respectively (128, 129, 133). The L gene expression is partially or completely dependent on the viral DNA replication (479). Our data thus far indicated that the suppression of key factors for type-I IFN production does not set in until after DNA synthesis by T953. We previously showed that blockage of T953 late gene expression eliminated the viral suppressive effect on type-I IFN induction (406). For this reason, we hypothesized that one or more late viral genes of T953 are involved in switching off phosphorylation of STAT2 molecules. In order to
investigate this phenomenon, we blocked the T953 viral late gene expression with a chemical inhibitor, phosphonoacetic acid (PAA) (Acros Organics, NJ). We then compared the cellular abundance and phosphorylation of STAT2 in T953-infected EECs in the presence or absence of PAA (300 μg/ml). The effectiveness of the PAA-mediated blockage of T953 was demonstrated by analysis of expression of a viral L protein gD, in comparison with the expression of viral IE protein. We observed that at 3 hpi, EHV-1 gD was not expressed either in the presence or absence of PAA treatment (data not shown). This was expected because gD is one of the L proteins of EHV-1 that is not synthesized during early infection. Consistent with previous findings, phosphorylated STAT2 was not detected in mock-infected EECs, but was readily detectable 30 min after rEqIFN-α treatment in the positive control. In the absence and presence of PAA, T953 failed to either induce or block the phosphorylation of STAT2 at 3 hpi and 6 hpi (Fig. 3.7.A and 3.7.B). In contrast with untreated cells, T953 infection in the presence of PAA was unable to prevent rEqIFN-α-induced phosphorylation of STAT2 at 12 hpi (Fig. 3.7.C). This data indicates that inhibition of STAT2 phosphorylation involves one or more late viral genes of T953, whose expression is blocked in the presence of PAA.

3.4 Discussion

In natural outbreaks, EHV-1 has a predilection for the epithelial cells of the upper respiratory pathway, but cell-associated virus can eventually be transmitted to EECs lining the blood vessels of the uterus and those of the CNS. When this happens, these cells can mount an immediate innate immune
response by activating the type-I IFN signaling cascade. EHV-1 is capable of employing a variety of ways to escape the early innate immune response at least \textit{in vitro} and presumably also \textit{in vivo}. Here, we explored some molecular mechanisms by which EHV-1 renders the host type-I IFN signaling ineffective during the infection process, using our already established EEC model.

Most viral intruders are recognized by PRRs such as the TLRs which initiate signaling cascades leading to type-I IFN production (361). The ability to immediately counteract recognition by these PRRs enables virus replication in host cells and facilitates an effective viral infection. Our study revealed that EHV-1 interferes with the host cell’s ability to recognize viral invasion by suppressing the expression of TLR3 and TLR4 mRNA by 6 and 12 hpi in EECs. Many viruses impede the expression of TLRs in order to escape the antiviral effect of type-I IFN. For instance, respiratory syncytial virus strain A2 and a measles virus strain block type-I IFN production in a plasmacytoid dendritic cell model and this is mediated by inhibition of both TLR7 and TLR9 (480). Since TLR3 recognizes dsRNA (458) while TLR4 recognizes viral glycoprotein (459), it is plausible that by reducing TLR3 and TLR4 expression in EECs, EHV-1 is able to evade cellular PAMP recognition, thereby enhancing its effective replication. Individual TLR signaling is dependent on a set of TIR domain-containing adaptors such as Myd88, TRIF, TIRAP/MAL or TRAM for a robust innate immune response including type-I IFN production (481). Another possibility is that by inhibiting TLR3 and TLR4 expression in EECs, EHV-1 may block the recruitment of these adaptor proteins thereby compromising the antigen-induced signal transduction.
needed for a potent type-I IFN response. Although our study did not investigate the effects of EHV-1 on cytosolic PRRs such as RIG-I-like receptors, we speculate, based on our TLR3 and TLR4 data, that the same EHV-1 viral factors similarly interfere with their sensitivity during infection.

Transcription factors involved in IFN production are activated when a virus is initially detected by PRR. Activated transcription factors subsequently translocate to the nucleus and interact with IFN promoter sequences, leading to the upregulation of IFN genes. It has been shown that herpesviruses and other viruses can down-regulate IRF3, IRF7 or IRF9 signaling to suppress IFN-α/β production (482-487). Recently, we determined that the molecular mechanism for EHV-1 suppression of type-I IFN induction involves disruption of the IRF-3 signaling pathways (402). Whether EHV-1 is able to directly affect the cellular abundance of IRF7 and IRF9 remained poorly understood. Here we show that consistent with our earlier results, EHV-1 significantly downregulated IRF7 mRNA by 12 hpi and IRF9 mRNA by 6 and 12 hpi in EECs. These are the same time-points in our previous studies where suppression of type-I IFN was observed following EHV-1 infection of EECs (31, 402, 406, 469). Since both IRF7 and IRF9 play significant roles along with IRF3 in the induction phase of type-I IFN production, it seems plausible that EHV-1 blocks type-I IFN induction in the infected cell by preventing the expression of these transcription factors. One of the limitations of our study is the lack of equine specific anti-TLR3, anti-TLR4, anti-IRF7, and anti-IRF9 antibodies. This hindered our ability to investigate whether EHV-1 effects on TLR3, TLR4, IRF7, and IRF9 gene expressions extend
to the respective translated protein components. We hope such reagents will become available for study of the effect of EHV-1 on TLR3, TLR4, IRF7, and IRF9 protein products during infection of EECs.

In this study, we found that EHV-1 disrupts the JAK/STAT signaling pathway by degrading the cellular levels of TYK2 at 12 hpi. Interestingly, EHV-1 exerted no significant effect on the cellular abundance of JAK1 at all studied time-points. Since TYK2 helps phosphorylate STAT2 (320), which in turn creates a docking site for STAT1 phosphorylation (324, 488), it is possible that EHV-1 specifically targets TYK2 to prevent the interaction between the two STAT proteins in order to block type-I IFN induction. Several other viruses have evolved mechanisms to prevent the activation of STAT2 proteins by TYK2. One such example is Epstein-Barr virus that negatively regulates TKY2 phosphorylation and IFN signaling in human B cells using a latent membrane protein 1 (489). Although we do not understand the exact mechanism employed by EHV-1 to degrade TYK2, we hypothesize that certain viral proteins may act to target TYK2 for proteosomal degradation as has been reported for human metapneumovirus (21).

As expected, our study revealed that EHV-1 prevents IFN-induced phosphorylation of the STAT1/STAT2 molecule by 12 hpi. EHV-1 seems to induce only the downregulation of STAT1 phosphorylation without affecting JAK1 whereas a reduced cellular abundance of TYK2 leads to the downstream inhibition of STAT2 phosphorylation in response to EHV-1 infection. One major consequence of the lack of phosphorylated STAT1/STAT2 is the exclusion of
these STAT molecules from the nucleus of EHV-1 infected EECs. Previously, we
demonstrated STAT1 occlusion from the nucleus in EHV-1 infected cells even in
the presence of an exogenous IFN stimulant (31). Our new data revealed that
EHV-1 was able to prevent STAT2 activation/translocation, thereby resulting in
sequestration in the nucleus. Since EHV-1 also inhibits IRF9 mRNA at this time
point, we believe that EHV-1 blocks the nuclear accumulation of the ISGF3
heterocomplex as one of the ways of preventing type-I IFN induction. The
efficient inhibition of type-I IFN production by EHV-1 may be a consequence of
coordinated cytoplasmic sequestration of STAT2 proteins or narrowing of nuclear
pore complex to occlude STAT2 into the nucleus during infection as has been
reported for Nipah virus (490). This finding further explains why EHV-1 is able to
prevent the transcription of ISGs as we previously reported (31). Our data also
suggest that despite inhibiting IFN-induced STAT1/STAT2 phosphorylation, EHV-
1 did not alter the total cellular level of STAT1/STAT2 during infection. These
data, including that of JAK1, add to the body of evidence that the inhibitory
effects of EHV-1 on the other key factors of type-I IFN signaling are not a result
of the global shutdown of host macromolecular synthesis during infection (491).
Our data revealed an elevated lamin A/C with EHV-1 infection when compared to
uninfected controls. In a recent study, Banati and co-workers also reported up-
regulated lamin A/C in Epstein-Barr virus immortalized lymphoblastoid cell lines
and in group III Burkitt lymphoma lines (492). An up-regulated lamin A/C, as seen
in our study, may facilitate viral gene transcription/replication in the nucleus of
EHV-1-infected cells. Although it appeared that the cytoplasmic portion of total
STAT2 was affected in virus-infected cells, densitometric analysis of Western blot images revealed no difference between total STAT2 in EHV-1-infected cells and uninfected controls (data not shown).

We showed that the ability to suppress key molecules of the type-I IFN response, including IFN-induced STAT2 phosphorylation, involves the \textit{de novo} synthesis of viral genes. UV-inactivation of EHV-1 abolished the suppressive effects that were present during active EHV-1 infection in EECs. Thus, it appears that viral DNA synthesis and some viral protein synthesis were required for the inhibition. Also, the only striking difference in the phenotypes of STAT2 phosphorylation in EECs infected with EHV-1 either in the presence or absence of PAA treatment occurred at 12 hpi. PAA-mediated blockage of late viral gene expression at this time-point resulted in no suppressive effect on IFN-induced STAT2 phosphorylation. Therefore, as we suggested previously (406), EHV-1 encodes one or more late viral genes that target host factors needed for type-I IFN response. These viral factors responsible for the inhibitory effect on host type-I IFN response remain to be identified. It has been reported that HSV-1 utilizes a late viral protein, UL41, to mediate inhibition of STAT1 phosphorylation (493). Another late protein, US3 (a serine-threonine kinase), of HSV-1 is also involved in the evasion of the type-I IFN response by interfering with the TLR3-mediated immune response (398, 399). EHV-1 encodes homologs of HSV-1 UL41 and US3 proteins (434). However, their effects in an equine host in the context of STAT2 phosphorylation and TLR3-mediated type-I IFN response are yet to be explored. Further studies are, therefore, required to elucidate whether
the roles of EHV-1 homologs of HSV-1 UL41 and US3 proteins are functionally conserved between both alphaherpesviruses.

Our results are contrary to the finding of Edington et al. (494) that EHV-1 induces a type-I IFN response in the horse. Also, Wagner et al. (495) showed that equine PBMCs infected with EHV-1 strains including the neuropathogenic strain Ab4 robustly secreted IFN-α at 48 hpi, and Holz et al. (496) detected IFN-α in nasal secretions from Ab4-infected horses on days 1 and 2 post infection; interestingly this was much reduced in horses that went on to develop EHM. On the other hand, IFN-α was not detected in cerebrospinal fluid of Ab4-infected horses (496). Our results also should be seen in the light of those of Soboll Hussey et al. (497) who found that in cultured equine respiratory epithelial cells (EREcs), there was no significant IFN-α/β production at 12 hpi but considerably more at 24 hpi. In another similar study, Poelaert et al. (498) reported that both the abortigenic EHV-1 and the neurovirulent EHV-1 competently induced IFN-α in equine respiratory mucosa and ERECs, although, the abortigenic strains show more anti-IFN effect. These differences in observed response may be characteristic of the particular model being used and also to the kinetics of the infectious process in each model relevant to pathogenicity. The correspondence of EECs with ERECs with regards to type-I production following EHV-1 infection has not been previously studied. We are currently investigating this effect to further understand whether the kinetics of type-I IFN response during EHV-1 infection correlates with cell type. We hypothesize that the virus-mediated IFN suppression effect does not operate on neighboring cells but only on the infected
cell. Also, the effect may be more consequential in some cell types (endothelial) than others. This effect may be more related to facilitating virus replication in the face of exogenous IFN stimulation as the IFN-α receptor also signals in part through the JAK/STAT pathway. Finally, we have shown that EHV-1 can repress host type-I IFN response by specifically targeting the sensitization and the induction phase of type-I IFN during infection. This information could be useful in identifying the specific viral protein involved in host type-I IFN inhibition enabling the development of a future therapeutic target against EHV-1. Therapies that would synergistically improve both host cells’ recognition of the virus and IFN signaling would be more beneficial in preventing field outbreaks of more devastating EHV-1 diseases.
## Table 3.1 Primers and probes for RT-PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequences (5'-3’) / Assay ID</th>
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<tbody>
<tr>
<td>EqRPLP0</td>
<td>Fwd: CTGATTACACCTTCCCACCTTGCT</td>
</tr>
<tr>
<td></td>
<td>Rev: AGCCACAAATGCAGATGGATCA</td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-AAGGCCTTGACCTTTTC-NFQ (406)</td>
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<tr>
<td>EqTLR3</td>
<td>Ec03467747_m1</td>
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</tr>
<tr>
<td>EqIRF7</td>
<td>AJBJX9T</td>
</tr>
<tr>
<td>EqIRF9</td>
<td>ARMFXC1F</td>
</tr>
</tbody>
</table>
Figures.

**Figure 3.1** Effect of EHV-1 infection on TLR mRNA transcription. Mock-infected EECs (M, solid bars) were either treated with 80 µg/ml of Poly-I:C for A or 10 µg/ml of LPS for B (both labelled as P, clear bars) or infected at an MOI of 5 with T953 in the presence (P+E1, diagonal-striped bars) or absence of P (E1, checker board bars). At indicated time-points, the cells were lysed and equine TLR3 (A) and TLR4 (B) mRNA were quantified by real-time RT-PCR as described in the text. Data were normalized to the levels of endogenous control equine RPLPO mRNA at the same time point. Each bar represents the mean and standard deviation from three independent experiments. Data were significant at P < 0.05 (*), P < 0.01 (**), and P < 0.0001 (****) while non-significant data is denoted by ns.

**Figure 3.2** Effect of EHV-1 infection on IRF mRNA transcription. EECs were either mock infected (M in solid bars) or Poly-I:C-treated (P in clear bars) or infected at an MOI of 5 with T953 in the presence (P+E1 in diagonal-striped bars) or absence of P (E1 in checker board bars). At indicated time-points, the cells were lysed and equine IRF7 (A) and IRF9 (B) mRNA were quantified by real-time RT-PCR as described in the text. Data were normalized to the levels of endogenous control equine RPLPO mRNA at the same time point. Each bar represents the mean and standard deviation from three independent experiments. **** shows statistical significance at P < 0.0001 while ns indicates non-significant data.
Figure 3.3  Effect of EHV-1 infection on cellular abundance of endogenous JAK1 and TYK2. EECs were either mock-infected (M) or infected with T953 (E1) at an MOI of 5 for 3 h (A), 6 h (B) or 12 h (C). The cells were then treated or left untreated (+/- in figure) with equine rEqIFN-α at 1000 IU/ml for 30 min before lysis at indicated time points. Abundance of total JAK1 and TYK2 was analyzed by Western blot and band intensities were normalized to the endogenous levels of β-actin. Densitometric analysis of Western blot images for JAK1 (D) and TYK2 (E) normalized against β-actin were done by ImageJ (NIH). The Western blot images were representative of 3 different blots from independent experiments.
Figure 3.4 Effect of EHV-1 infection on IFN-induced STAT1/STAT2 phosphorylation. EECs were either mock-infected (M) or infected with T953 (E1) at an MOI of 5 and cells were either treated or left untreated (+/- in figure) with equine rEqIFN-α at 1000 IU/ml for 30 min before lysis. Abundance of phosphorylated versus total STAT1 at 12 h (A), and phosphorylated versus total STAT2 at 3 h (B), 6 h (C), or 12 h (D) were then quantified using Western blot analysis. Membranes were stripped and reprobed with β-actin as a control for equal loading of samples. Densitometric analysis of Western blot images for phosphorylated STAT1, total STAT1 (E), and phosphorylated STAT2, total STAT2 (F) normalized against β-actin were done by ImageJ (NIH). The Western blot images were representative of 3 different blots from independent experiments.
Figure 3. Effect of EHV-1 infection on nuclear accumulation of STAT2. EECs, grown on coverslips in 24-well plates were either infected with T953 at an MOI of 3 or mock-infected. The cells were either stimulated with equine rEqIFN-α at 1000 IU/ml or equal volume of plain media for 30 min prior to fixation in 4% PFA at 12 hpi. Cells were stained with anti-STAT2 (1:100 dilution) and anti-EHV-1 gC antibody (1:200 dilution). N ≈ 300 cells from different fields were examined for each treatment using an inverted fluorescent microscope. (A) Mock-infected EECs treated with plain media and stained for STAT2. (B) Mock-infected cells stimulated with equine rEqIFN-α and stained for STAT2. (C) T953 infected cells stained for STAT2. (D) T953 infected cells stimulated with equine rEqIFN-α and stained for STAT2. 4,6-diamidino-2-phenylindole (DAPI: blue) served as a counterstain. Solid arrowheads indicate representative cells without nuclear STAT2 translocation whereas open arrowheads indicate representative cells with nuclear STAT2 translocation. Scale bar: 50 µm. (E) Quantification of nuclear STAT2 intensity of IF images using Nikon NIS-Elements software as described in Materials and Methods. (F) EECs were either mock infected (M) or infected with T953 (E1) at an MOI of 3 and cells were either treated or left untreated (+/- in figure) with equine rEqIFN-α (1000 IU/ml) for 30 min before fractionation into nuclear (N) or cytoplasmic (C) compartment. Separated cellular fractions were probed for phosphorylated STAT2 and total STAT2 to determine their cellular distribution following T953 infection. Lamin A/C and MEK1/2 were used as nuclear and cytoplasmic loading controls respectively.
Figure 3. 6 Effect of UV-inactivation on type-I IFN molecules. Mock-infected EECs (M, solid bars) were either treated with 80 µg/ml of Poly-I:C for A, C, and D or 10 µg/ml of LPS for B (both labelled as P, clear bars) or infected with UV-inactivated T953 in the presence (P+E1, diagonal-striped bars) or absence of P (E1, checker board bars) as described in the text. At indicated time-points, the cells were lysed and equine TLR3 (A), TLR4 (B), IRF7 (C), and IRF9 mRNA (D) were quantified by real-time RT-PCR. Data were normalized to the levels of endogenous control equine RPLPO mRNA at the same time point. Non-significant data is denoted by ns. (E) EECs were either mock-infected (M) or infected with UV-inactivated T953 (E1) at an MOI of 5 and cells were either treated or left untreated (+/- in figure) with 1000 IU/ml of equine rEqIFN-α for 30 min prior to lysis. Following 12 hpi, cells were lysed and equal amounts of proteins were separated on 10% SDS-PAGE and probed using phospho-STAT2 or STAT2. Images were representative of 3 independent biological experiments.
Figure 3. Effect of viral late gene blockage on STAT2 activation. EECs were infected with T953 (E1) either in the presence (left columns) or absence of PAA (300 µg/ml). Mock-infected EECs (M) were either treated or left untreated (+/- in figure) with equine rEqIFN-α at 1000 IU/ml for 30 min before lysis. Abundance of phosphorylated versus total STAT2 at 3 h (A), 6 h (B), or 12 h (C) were then quantified using Western blot analysis. Membranes were stripped and reprobed with β-actin as a control for equal loading of samples. As controls for effective PAA-mediated blockage of T953 L proteins, both the EHV-1 IE and EHV-1 gD were also quantified. The Western blot images were representative of 3 different blots from independent experiments.
CHAPTER 4: THE EFFECT OF EQUINE HERPESVIRUS TYPE 4 ON TYPE-I INTERFERON SIGNALING MOLECULES.

Abstract

Equine herpesvirus type 4 (EHV-4) is mildly pathogenic but is a common cause of respiratory disease in horses worldwide. We previously demonstrated that unlike EHV-1, EHV-4 is not a potent inducer of type-I IFN and does not suppress that IFN response, especially during late infection, when compared to EHV-1 infection in equine endothelial cells (EECs). Here, we investigated the impact of EHV-4 infection in EECs on type-I IFN signaling molecules at 3, 6, and 12 hpi. Findings from our study revealed that EHV-4 did not induce nor suppress TLR3 and TLR4 expression in EECs at all the studied time points. EHV-4 was able to induce variable amounts of IRF7 and IRF9 in EECs with no evidence of suppressive effect on these important transcription factors of IFN-α/β induction. Intriguingly, EHV-4 did interfere with the phosphorylation of STAT1/STAT2 at 3 hpi and 6 hpi, less so at 12 hpi. An active EHV-4 viral gene expression was required for the suppressive effect of EHV-4 on STAT1/STAT2 phosphorylation during early infection. One or more early viral genes of EHV-4 are involved in the suppression of STAT1/STAT2 phosphorylation observed during early time points in EHV-4-infected EECs. The inability of EHV-4 to significantly down-regulate key molecules of type-I IFN signaling may be related to the lower severity of pathogenesis when compared with EHV-1. Harnessing this knowledge may prove useful in controlling future outbreaks of the disease.

Key words: EHV-4, STAT1, STAT2, innate immunity, type-I interferon.
4.1 Introduction

Equine herpesvirus 4 (EHV-4) is a ubiquitous virus relevant for its ability to cause clinically significant upper respiratory tract (URT) disease in horse populations worldwide. This virus, along with EHV-1, has been found for over 80 years to be the most common herpesviruses impeding successful breeding, competition, and recreational horse industries all around the world (499). EHV-4 belongs to the genus Varicellovirus of the subfamily Alphaherpesvirinae (74). The virus was considered as subtype 2 of EHV-1 and known as the same virus until 1981 when DNA sequence information indicated otherwise (433, 500). EHV-4 has a genome size of about 146-kbp and encodes 76 ORF, with three of those genes duplicated (81). The genomic architecture of EHV-4 comprises a linear double-stranded, DNA organized into a long unique segment (UL, 112,398 bp) flanked by a short inverted repeat sequence (TRL/IRL, 27 bp) and a short unique segment (US, 12,789 bp) that is flanked by a large inverted repeat sequence (TRS/IRS, 10,178 bp) (81, 499). The level of nucleotide identity between EHV-1 and EHV-4, within individual homologous genes, ranges from 55-84% while the amino acid sequence identity ranges from 55-96% (81, 82). Despite the close relatedness of EHV-4 to EHV-1, the genetic differences between the two viruses are sufficient to affect their host range and disease phenotypes. EHV-4 mostly infect the URT of horses causing respiratory syndromes, whereas EHV-1 has a more diverse host range and causes pathologies including respiratory disease, abortion and equine herpesviral myeloencephalopathy (EHM) (105).
Unlike EHV-1, EHV-4 has a streamlined host range and the virus can efficiently infect only equine cells; it replicates only poorly in a few other cell lines (501). Lytic infection by EHV-4 is thought to mainly occur in the epithelial cells of the URT, although equine endothelial cells (EEC) are also permissive to EHV-4 infection (5). Alphaherpesviruses have been demonstrated to enter different cell types by different pathways that, mostly, utilize similar viral glycoproteins, namely, glycoprotein D (gD), gB, gH, and gL, in conjunction with host cellular receptors and coreceptors (502-506). The major viral component that defines the differential cellular tropism between EHV-1 and EHV-4 has been found to be gD. An exchange of gD of EHV-1 for that of EHV-4 was able to enhance EHV-4 entry in an originally non-permissive cell (507). Unlike many alphaherpesviruses that enter endothelial cells via viral envelope fusion with the plasma membrane, EHV-4 does so by an endocytic pathway which is reportedly dependent on dynamin-II, cholesterol, caveolin-1, and tyrosine kinase (507).

The detailed pathogenesis of EHV-4 has not been fully uncovered but the infection mirrors that of EHV-1 at the initial stage in which the virus infects the epithelial cells of the URT and its associated lymphoid system. However, the pathogenicity, extent of virus spread and invasiveness in an infected horse are not as severe when compared to EHV-1 infection. The sequence similarity of EHV-4 with EHV-1 makes a detailed examination of EHV-4 pathogenesis useful, as this can provide clues to the molecular basis of pathogenesis of both viruses. Usually, the extent of EHV-4 infection and viral cell-to-cell spread does not occur beyond the infection of regional lymph nodes of the URT (206). The virus is
normally unable to attain the level of viremia in circulating lymphocytes needed to initiate the process of abortion and neurologic syndromes seen in many cases of EHV-1 infection. However, cell-associated viremia as a result of EHV-4 has been reported (508) and in rare cases, EHV-4-induced abortion has been documented (509). The ability of EHV-4 to induce abortion, although rare, suggests that the virus can sometimes overcome host immune activity that normally limits the infection process. Over the years, many studies have focused on the evasion of host immune protection by EHV-1 in equine hosts. However, in comparison, relatively little is known about the immune evasion strategies of EHV-4. Recently, we were able to demonstrate that EHV-4 reduced type-1 IFN production at the early onset of infection but lost this ability over time (469). Here, we investigated the impact of EHV-4 on host type-1 IFN signaling molecules in order to gain insight into the immune evasion strategies of EHV-4 during infection using an EEC model.

4.2 Materials and methods

4.2.1 Cells and viruses

Equine pulmonary artery endothelial cells (EECs; (437)) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc.) with 10% iron-supplemented bovine calf serum (BCS, Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin-streptomycin (Life Technologies, Carlsbad, CA), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 200 mM L-glutamine (Life Technologies, Carlsbad, CA) in a humidified incubator at 37 °C with 5% CO₂. The EHV-4 strain T445 (hereafter EHV-4) was previously isolated and archived
by the late Dr. George P. Allen at the Maxwell H. Gluck Equine Research Center, University of Kentucky. Virus stocks of EHV-4, were prepared by infection of 90 to 100% confluent EECs at a multiplicity of 0.5 in DMEM in the absence of BCS as described (469). After the virus has produced nearly 100% cytopathic effect (CPE), the tissue culture fluid (TCF) containing the virus was freeze-thawed three times and clarified at 2000 x g for 30 min at 4°C, filtered through 0.45 μm cellulose acetate membrane filters (Thermo Scientific Nunc, Pittsburgh, PA) and purified by ultracentrifugation at 100,000 x g for 4 h at 4°C through a 20% sucrose cushion. The virus pellet was re-suspended in DMEM with 2% BCS, sonicated briefly, aliquoted in 100μl volumes and stored at -80°C until further use. The infectious virus titer was determined by plaque assay in EECs as described (438).

4.2.2 Viral infections

EECs were seeded in 6-well culture plates (Corning, NY) 48 h prior to infection to obtain more than 90% confluency at the time of infection. Monolayer cells were thereafter infected with EHV-4 at an MOI of 5 for 1 h in a humidified incubator at 37 °C with 5% CO₂. In parallel, cells were either mock-infected with virus diluent (negative control) or treated with 80 μg/ml of polyinosinic acid: polycytidylic acid (Poly-I:C) (positive control). The cells were then incubated with complete growth medium for 3 h, 6 h, and 12 h respectively. Cell lysates were collected at these time points to evaluate target mRNAs and proteins using RT-PCR and western blot assay respectively. All experiments were performed in duplicate and repeated on three independent days.
4.2.3 RNA extraction and real-time RT-PCR assay

Total cellular RNA was extracted using the QiaAmp RNeasy plus mini kit (Qiagen Inc. Valencia, CA) from EHV-4 infected and control EECs at 3 h, 6h, and 12 h respectively according to the manufacturer’s protocol. The quantity and quality of the cellular RNA were examined by OD_{260}/OD_{280} measurement using the Synergy H1 hybrid plate reader (Biotek, Winooski, VT). One microgram of total cellular RNA was reverse transcribed as described (440) using 0.5 µg oligo dT primer. Equal amounts of cDNA were used for the transcription analysis of different genes by TaqMan real-time PCR using primers and probes (Thermo Scientific, Pittsburgh, PA) in a ViiA™ 7 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Details of the primers and probes used in this study are provided in Table 1. All reactions were performed in duplicate. The RT-PCR data were normalized using the equine ribosomal protein large P0 (RPLP0) transcript as an endogenous control. The PCR efficiency for all reactions was assessed by LinReg software (441). Fold changes in the gene expression were calculated using the comparative ΔΔCT method for relative quantification (RQ) (442), using the average Ct value of mock-infected samples for each individual gene as the calibrator.

4.2.4 Western blot analysis

Confluent EECs were infected with EHV-4 at an MOI of 5. At 30 minutes prior to the end of experiment, infected cells were treated with recombinant equine interferon-α (rEqIFN-α) at 1000IU/ml to stimulate phosphorylation of STAT proteins following established protocols (31, 470). Cells were then washed
in cold PBS and solubilized in RIPA lysis buffer system (Santa Cruz, Dallas, TX) enriched with phosphatase inhibitor (Thermo Scientific, Pittsburgh, PA) on ice. Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific, Pittsburgh, PA) and measured using the Synergy H1 hybrid plate reader (Biotek, Winooski, VT). Equal amount of proteins were then separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (BioRad, Hercules, CA). Membranes were blocked with 5% milk, 0.1% Tween-20 in Tris-buffered saline (TBS), and incubated overnight with the appropriate primary antibodies according to the manufacturer’s direction. The membranes were then washed and incubated with corresponding secondary antibody for 1 h at room temperature (RT). Protein bands were detected by enhanced chemiluminescence (Thermo Scientific, Pittsburgh, PA) and imaged immediately using Azure c600 (Azure Biosystems, Dublin, CA).

4.2.5 Antibodies and other reagents

Rabbit anti-STAT1 and rabbit anti-STAT2 primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti-β-actin primary antibody, rabbit anti-phospho-STAT1 primary antibody, and goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP) were purchased from Cell Signaling Technology (Beverly, CA). Rabbit anti-phospho-STAT2 primary antibody was purchased from Rockland Immunochemicals Inc. (Limerick, PA). Polyninosinic acid: polycytidylic acid (Poly-I:C) and lipopolysaccharide (LPS) were purchased from Invivogen (San Diego,
CA) and Phosphonoacetic acid (PAA) was purchased from Fisher Scientific (Pittsburgh, PA). Recombinant equine interferon (rEqIFN-α) was purchased from Kingfisher Biotech, Inc. (Saint Paul, MN).

4.2.6 Statistical analysis

Data from the study were analyzed by ANOVA with pairwise multiple comparison procedures by Tukey's HSD test using GraphPad Prism version 6.04 (GraphPad Software, Inc., La Jolla, CA). P-Values less than 0.05 were considered to be statistically significant.

4.3 Results

4.3.1 Effect of EHV-4 on TLR expression in EECs

One of the mechanisms by which the innate immune system senses impending invasion by pathogenic microorganisms is through cellular Toll-like receptors (TLR) which recognize conserved small molecular motifs known as pathogen-associated molecular patterns (PAMP) (510). We first evaluated the impact of EHV-4 infection on TLR3 and TLR4, based on their reported prominent roles in the viral replication cycle (458, 511) and their relative abundance in EECs. The kinetics of induction of TLR3/TLR4 mRNA following EHV-4 infection in EECs revealed a manner similar to the expression observed in mock-infected cells at 3, 6, and 12 hpi (Fig. 4.1A and Fig. 4.1.B). In the presence of either TLR3 agonist (Poly-I:C) or TLR4 agonist (LPS), EHV-4 was unable to statistically significantly reduce TLR3/TLR4 mRNA at all studied time-points. These findings suggest that EHV-4 can neither induce nor inhibit TLR3/TLR4 expression in EECs at the studied time-points. An antagonistic effect of EHV-4 on TLR3 and
TLR4, part of a key host cell innate immune sensing apparatus, was not evident which is in accord with our previous finding of elevated IFN-β levels in EHV-4 infected EECs at later time points (469).

4.3.2 Effect EHV-4 on interferon (IFN) transcription factors (IRF)

Interferon regulatory factors (IRF) 3, 7, and 9 are essential factors needed for efficient transcription of diverse IFN-α/β genes which in turn promote robust antiviral immune responses (309, 483, 512, 513). We examined the effect of EHV-4 on IRF7 and IRF9 mRNA following infection in EECs. EHV-4 induction of IRF7 mRNA was similar to what was observed in mock-infected cells at 3, 6, and 12 hpi (Fig. 4.2.A). The virus was also unable to statistically significantly alter IRF7 mRNA in cells stimulated with Poly-I:C at these time-points. The pattern of induction of IRF9 mRNA following EHV-4 infection was slightly different. Although not statistically significant, EHV-4 appeared to induce IRF9 mRNA at 3 hpi, however this induction had declined at 6 and 12 hpi respectively (Fig. 4.2.B). In the presence of Poly-I:C, EHV-4 was unable to statistically significantly inhibit the expression of IRF9 mRNA at all three time-points.

4.3.3 Effect of EHV-4 on STAT1/STAT2 phosphorylation

Activated STAT1 in conjunction with activated STAT2 and IRF9 form an IFN activated transcription factor, ISGF3, which is a heterocomplex involved in IFN-α/β gene induction (514-516). We recently demonstrated that EHV-1 has the ability to interfere with the phosphorylation and activation status of STAT1/STAT2 in infected EECs ((31); Oladunni et al., manuscript submitted). To investigate whether EHV-4 may interfere with the cellular abundance and activation status of
STAT1/STAT2, EECs were infected at an MOI of 5 and then treated with 1000 IU/ml of rEqIFN-α for 30 min prior to cell lysis for western blot assay. Findings from our study revealed that EHV-4 infection failed to induce the phosphorylation of STAT1/STAT2 at 3 hpi (Fig. 4.3.A and Fig. 4.3.D). The virus also reduced phosphorylation of STAT1/STAT2 when cells were stimulated with exogenous IFN at this time-point. By 6 hpi, EHV-4 had induced a small but detectable level of STAT1/STAT2 phosphorylation (Fig. 4.3.B and Fig. 4.3.E). However, virus infection also suppressed phosphorylation of STAT1/STAT2 that was stimulated by exogenous IFN at 6 hpi. By 12 hpi, EHV-4 infection had stimulated phosphorylation of STAT1/STAT2 in infected EECs to levels comparable to exogenous IFN stimulation (Fig. 4.3.C and Fig. 4.3.F). At 12 hpi, there was little sign of viral suppression of exogenous IFN-stimulated phosphorylation of STAT1/STAT2. The endogenous level of total STAT1/STAT2 remained unchanged at all the three time points. Findings from our study revealed a trend of a progressive increase in the cellular abundance of phosphorylated STAT1/STAT2 in EHV-4-infected cells either in the presence or absence of an exogenous IFN treatment which differs from the pattern observed in EHV-1 infection (31; Oladunni et al., manuscript submitted).

4.3.4 Effect of UV-inactivated EHV-4 on STAT1/STAT2 phosphorylation

The data above indicate that EHV-4 infection exerted a suppressive effect on endogenous levels of phosphorylated STAT1/STAT2 early in the infection process. For this reason, we next tested the hypothesis that the suppression of phosphorylated STAT1/STAT2 is dependent on an active viral replication of
EHV-4 in EECs. We performed similar experiments as above, using UV-inactivated EHV-4. Briefly, purified EHV-4 was UV-inactivated in a 33 mm petri dish placed 10 cm from a 40-watt UV lamp (UV Stratalinker 1800, CA) for 30 min. The inactivation of UV-treated EHV-4 was demonstrated by the absence of cytopathic effect when this was used to infect RK-13 cells. EECs were then infected with UV-inactivated EHV-4 at an equivalent dilution as the wild type (WT) EHV-4. Total cell lysates were then prepared for western immunoblotting. Mock-infected cells stimulated with 1000 IU/ml of rEqIFN-α were included as the positive control. Following 3hpi, EECs infected with UV-inactivated EHV-4 induced a level of phosphorylated STAT1/STAT2 similar to that of mock-infected cells (Fig. 4.4.A and Fig. 4.4.D). The UV-inactivated virus also failed to downregulate phosphorylated STAT1/STAT2 when cells were co-incubated with exogenous IFN at 3 hpi. Similarly, EECs infected with UV-inactivated EHV-4 neither induced nor suppressed phosphorylation of STAT1/STAT2 in the presence of rEqIFN-α at 6 hpi (Fig. 4.4.B and Fig. 4.4.E). This trend continues at 12 hpi. EECs infected with UV-inactivated EHV-4 induced a similar level of phosphorylated STAT1/STAT2 when compared with mock-infected cells (Fig. 4.4.C and Fig. 4.4.F). The UV-inactivated EHV-4 did not hinder the phosphorylation of STAT1/STAT2 in the presence of an exogenous IFN at 12 hpi. UV-inactivated EHV-4 infection also did not alter the cellular abundance level of total STAT1/STAT2 at 3, 6, and 12 hpi. These findings suggest that active viral replication is required for EHV-4 to affect the cellular abundance of
phosphorylated STAT1/STAT2 in EECs during an infection, and that proteins associated with the infecting virion particles are not responsible.

4.3.5 Effect of EHV-4 late gene expression on STAT1/STAT2 phosphorylation

The suppression of phosphorylated STAT1/STAT2 exhibited in EHV-4-infected cells was prominent at 3 hpi and 6 hpi, but not at 12 hpi. This pattern suggests a mechanism involving one or more viral early genes. To rule out dependence on EHV-4 late gene expression, we used a chemical inhibitor, Phosphonoacetic acid (PAA) (Acros Organics, NJ), to block the EHV-4 late gene expression as previously described (406, 517). We then evaluated the cellular abundance and phosphorylation of STAT1/STAT2 in EECs infected with EHV-4 in the presence or absence of PAA at different time-points by western blot. In the presence of PAA, EHV-4 failed to induce phosphorylated STAT1/STAT2 at 3 hpi (Fig. 4.5.A and Fig. 4.5.D). The PAA-treated EHV-4 also exerted a downregulating effect on phosphorylated STAT1/STAT2 in the presence of exogenous IFN at 3 hpi. Following 6 hpi, PAA-mediated blockage of EHV-4 late gene expression induced a modest level of phosphorylated STAT1/STAT2 in infected EECs (Fig. 4.5.B and Fig. 4.5.E). Also, at this time-point, PAA-treated EHV-4 suppressed the phosphorylation of STAT1/STAT2 in infected cells in a manner observed with the WT virus. At 3 and 6 hpi, the patterns observed in untreated and PAA-treated EHV-4-infected EECs were similar, indicating the involvement of early rather than late viral factors. Interestingly, PAA-treated EHV-4 induced a small level of phosphorylated STAT1/STAT2 but reduced the phosphorylation of STAT1/STAT2 stimulated by rEqIFN-α at 12 hpi (Fig. 4.5.C
and Fig. 4.5.F). The endogenous level of total STAT1/STAT2 remained unchanged at all the three time-points studied. These data suggest to us that possibly some late viral genes of EHV-4 have a counteracting effect on viral suppression of phosphorylated STAT1/STAT2. This finding is in contrast to that of EHV-1 virus that utilizes late viral genes to repress STAT1/STAT2 phosphorylation in infected EECs ((31); Oladunni et al., manuscript submitted).

4.4 Discussion

The primary cellular targets of most respiratory virus infections are epithelial cells of the respiratory tract. They play an active role in the production and release of IFN-α/β which mediates antiviral innate immune responses (518) to inhibit viral replication in infected cells (519). Within the lower respiratory tract, alveolar epithelial cells are in close proximity to the underlying endothelium which is thus susceptible to infection by virus particles produced by infected and damaged epithelial cells (520). Previously we have demonstrated that there is a difference in the induction kinetics of IFN-β between EHV-1 and EHV-4 in EECs (469), a cell line related to the endothelial cells lining all blood vessels. Here, we explored the influence of EHV-4 infection on important host cell signaling molecules needed for type-I IFN production using our already established EEC model.

Our present study revealed that EHV-4 lacked the potency to stimulate TLR3 and TLR4 transcription following infection in EECs. Since TLR3 and TLR4 expression is not up-regulated, it is possible IFN induction by EHV-4 utilizes other sensors than the TLR family, such as those triggered by cytoplasmic
recognition of viral replication; retinoic acid-inducible gene I (RIG-I), or melanoma differentiation-associated protein 5 (MDA-5) to initiate type-I IFN signaling pathways in EECs. All DNA viruses, including EHV-4, are known to produce dsRNA by convergent transcription and thus could activate IFN-inducible dsRNA-dependent protein kinase R (PKR), and possibly RIG-I or MDA-5 (521). In addition, EHV-4 did not have the capacity to suppress TLR3 and TLR4 transcription. This is unlike most other herpesviruses. For example, we demonstrated that the molecular mechanism of suppression of type-I IFN induction by EHV-1, a virus closely related to EHV-4, involves the virus first downregulating TLR3 and TLR4 transcription in EECs (Oladunni et al., manuscript submitted). Also, TLR4 mRNA levels and protein expression are significantly downregulated upon primary Kaposi sarcoma-associated herpesvirus (KSHV) infection of human lymphatic endothelial cells (522). EHV-4 did not inhibit TLR3 and TLR4 expression, so we do not envisage an antagonistic response to the effectiveness of synthetic TLR agonists like Poly-I:C were such to be administered as immune adjuvants when vaccinating against EHV-4 infection.

Transcription factors involved in IFN production are activated when a virus is initially detected. Activated transcription factors, such as IRF7, subsequently translocate to the nucleus and interact with IFN promoter sequences, leading to the upregulation of IFN genes (523). Data from our study showed that EHV-4 was unable to affect IRF7 transcription. These data suggest that during EHV-4 infection of EECs, the endogenous level of IRF7 remained fairly abundant and
capable of transactivating type-I IFN inducible genes. Our finding is in contrast to that of Zhu et al., who found that the ORF45 of KSHV interacts with IRF-7 and inhibits its phosphorylation and nuclear accumulation (523). It is also dissimilar to herpes simplex virus type1 (HSV-1) which blocks IFN regulatory factor IRF3- and IRF7-mediated activation of IFN-stimulated genes using its ICP0 RING finger domain (524). These examples are pointers to the anti-IFN mechanisms utilized by other herpesviruses to promote virus replication in their respective hosts.

EHV-4, however, apparently lacks a similar capacity due to its inability to inhibit IRF7 which is required, together with IRF3, for inducing type-I IFN genes. Our data also showed that EHV-4 was unable to downregulate IRF9 mRNA when cells were stimulated with Poly-I:C at all time points. Degradation of the cellular level of IRF9 and subsequent blocking of its nuclear translocation to prevent the formation of IFN stimulated gene factor3 (ISGF3) complex is an IFN-evasive mechanism utilized by many other herpesviruses (486, 487). Since both IRF7 and IRF9 play significant roles in the induction phase of type-I IFN production, we speculate that the inability of EHV-4 to downregulate these two key transcription factors may account for its lack of suppressive effect on type-I IFN in EECs (469).

Importantly, EHV-4 failed to induce any significant phosphorylation of STAT1/STAT2 early during infection at 3 h. At this same time-point, the virus had statistically significantly suppressed the phosphorylation of STAT1/STAT2 when cells were co-incubated with exogenous IFN. However, our data did show that induction of phosphorylation of STAT1/STAT2 in response to EHV-4 infection...
increased over time while the viral suppressive effect on phosphorylated
STAT1/STAT2 by exogenous IFN was lost as infection progressed. We believe
that the inability of EHV-4 infected cells to induce STAT1/STAT2 phosphorylation
at both 3 and 6 hpi even in the presence of exogenous IFN will down-regulate
IFN induction at early time-points, as we previously observed (469). We also
showed that the ability to inhibit the phosphorylation of STAT1/STAT2 by EHV-4
requires de novo viral DNA synthesis. UV-inactivation of EHV-4 abolished the
suppressive effects on STAT1/STAT2 phosphorylation that were profoundly
present during the early onset of WT virus infection at 3 and 6 h. The striking
difference in the phenotypes of STAT1/STAT2 phosphorylation in EECs infected
with EHV-4 either in the presence or absence of PAA treatment occurred at 12
hpi. Data suggested that one or more early viral genes of EHV-4 were needed for
suppression of STAT1/STAT2 phosphorylation, but by the later period this
activity has worn off.

One major shortfall of our study is our inability to demonstrate whether
EHV-4 alters the protein levels of studied TLRs and IRFs due to lack of equine-
specific antibodies. We hope that future studies will be able to address this
limitation to uncover the impact of EHV-4 on post-transcriptional levels of these
factors. The inability of EHV-4 to exert a negative influence on key molecules of
type-I IFN response may also be directly related to its reduced pathogenicity
when compared to EHV-1 explaining why EHV-4 infection is mostly restricted to
the URT. In a different study, Vandekerckhove et al., (215) reported a reduced
lateral spread of EHV-4 in nasal mucosal epithelium when compared to EHV-1
suggesting the inability of EHV-4 to inhibit the antiviral effects of type-I IFNs in mucosal epithelial cells. This striking difference in the pathogenic potential between EHV-1 and EHV-4 provides additional evidence for the lesser severity of respiratory signs observed, *in vivo*, during EHV-4 infection (105, 525, 526).
Table 4. Primers and probes for RT-PCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequences (5’-3’) / Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>EqRPLP0</td>
<td>Fwd: CTGATTACACCTTCCCACTTGCT</td>
</tr>
<tr>
<td></td>
<td>Rev: AGCCACAAATGCAGATGGATCA</td>
</tr>
<tr>
<td></td>
<td>Probe: FAM-AAGGCCTTGACCTTTTC-NFQ</td>
</tr>
<tr>
<td></td>
<td>(406)</td>
</tr>
<tr>
<td>EqTLR3</td>
<td>Ec03467747_m1</td>
</tr>
<tr>
<td>EqTLR4</td>
<td>Ec03468994_m1</td>
</tr>
<tr>
<td>EqIRF7</td>
<td>AJBJX9T</td>
</tr>
<tr>
<td>EqIRF9</td>
<td>ARMFXCF</td>
</tr>
</tbody>
</table>
Figures.

Figure 4.1 Effect of EHV-4 infection on TLR mRNA transcription. Mock-infected EECs (M, solid bars) were either treated with 80 µg/ml of Poly-I:C for A or 10 µg/ml of LPS for B (both labelled as P, clear bars) or infected at an MOI of 5 with EHV-4 in the absence (E4, checker board bars) or presence of P (P+E4, diagonal-striped bars). At indicated time-points, the cells were lysed and equine (A) TLR3 and (B) TLR4 mRNA were quantified by real-time RT-PCR as described in the text. Data were normalized to the levels of endogenous control equine RPLPO mRNA at the same time point. Each bar represents the mean and standard deviation from three independent experiments. Non-significant data is denoted by ns.

Figure 4.2 Effect of EHV-4 infection on IRF mRNA transcription. EECs were either mock infected (M in solid bars) or Poly-I:C-treated (P in clear bars) or infected at an MOI of 5 with EHV-4 in the absence (E4, checker board bars) or presence of P (P+E4, diagonal-striped bars). At indicated time-points, the cells were lysed and equine (A) IRF7 and (B) IRF9 mRNA were quantified by real-time RT-PCR as described in the text. Data were normalized to the levels of endogenous control equine RPLPO mRNA at the same time point. Each bar represents the mean and standard deviation from three independent experiments. Non-significant data is denoted by ns.
Figure 4.3  Effect of EHV-4 infection on IFN-induced STAT1/STAT2 phosphorylation. EECs were either mock-infected (M) or infected with EHV-4 (E4) at an MOI of 5 and cells were either treated or left untreated (+/- in figure) with equine rEqIFN-α at 1000 IU/ml for 30 min before lysis. Abundance of phosphorylated versus total STAT1 at (A) 3 h, (B) 6 h, and (C) 12 h or phosphorylated versus total STAT2 at (D) 3 h, (E) 6 h, and (F) 12 h were then quantified using western blot analysis. Membranes were stripped and reprobed with β-actin as a control for equal loading of samples. The western blot images were representative of 3 different blots from independent experiments.
Figure 4. Effect of UV-inactivated EHV-4 on STAT1/STAT2 phosphorylation. EECs were either mock-infected (M) or infected with UV-inactivated EHV-4 (E4) at an MOI of 5 and cells were either treated or left untreated (+/- in figure) with 1000 IU/ml of equine rEqIFN-α for 30 min prior to lysis. Abundance of phosphorylated versus total STAT1 at (A) 3 h, (B) 6 h, and (C) 12 h or phosphorylated versus total STAT2 at (D) 3 h, (E) 6 h, and (F) 12 h were then quantified using western blot analysis. Membranes were stripped and reprobed with β-actin as a control for equal loading of samples. Images were representative of 3 independent biological experiments.
Figure 4.5  Effect of viral late gene blockage of EHV-4 on STAT1/STAT2 phosphorylation. EECs were infected with EHV-4 (E4) either in the presence (left columns) or absence of PAA (300 µg/ml). Mock-infected EECs (M) were either treated or left untreated (+/- in figure) with equine rEqIFN-α at 1000 IU/ml for 30 min before lysis. Abundance of phosphorylated versus total STAT1 at (A) 3 h, (B) 6 h, and (C) 12 h or phosphorylated versus total STAT2 at (D) 3 h, (E) 6 h, and (F) 12 h were then quantified using western blot analysis. Membranes were stripped and reprobed with β-actin as a control for equal loading of samples. The western blot images were representative of 3 different blots from independent experiments.
CHAPTER 5: GENERAL DISCUSSION

5.1 Summary

Equine herpesvirus-1 (EHV-1), a DNA virus under the genus *Varicellovirus*, subfamily *alphaherpesvirinae*, is one of the most important and prevalent viral pathogens of horses and a major threat to the equine industry (499). Features of this viral infection involves the destruction of either the epithelial cells of the URT or the endothelial cells lining the small blood vessels of the pregnant uterus or the CNS which leads to outbreaks of sporadic abortions or CNS disorders as sequelae of an initial respiratory illness in affected horses (456, 527, 528). Prior research in the field has overwhelmingly focused on the impacts of the EHV-1 on the adaptive arm of the immune system of an infected horse. Certainly, such studies provide valuable insights, as the typical immune response to an intracellular pathogen requires a strong cell-mediated and humoral-immunity to clear the invading pathogens. However, such studies fail to provide a nuanced understanding of the distinct roles of an important arm of innate immunity- the type-I IFN system, which act as the ‘first responder’ of the host against an invading viral pathogen. EHV-1, like many other successful viruses, can circumvent this important host innate immunity but yet, only few studies have looked into the direct impacts of the virus infection on the host type-I IFN response. Here, our goal was to examine the alterations in the biology of the host type-I IFN response during equine alphaherpesviruses’ infection using a relevant cell culture model (EEC) chosen to attempt to mimic natural infection of the blood vessels of the uterus/CNS.
5.2 Major findings

5.2.1 Pathogenic EHV subtypes have inherent ability to shut down type-I IFN response in host cells.

Field outbreaks of EHV-1 vary in pathogenicity, and some but not all field isolates are highly neuropathologic (246, 411, 527). As a result, isolates of EHV-1 that exist in the field can be broadly classified as neuropathogenic and non-neuropathogenic, based on presence of a DNA_{pol} SNP which influences whether a horse develops a neurologic syndrome or not. Although it is believed almost all strains of EHV-1 can induce abortion in pregnant mares, in general the strains with an A_{2254} → G_{2254} mutation in the viral DNA_{pol} leading to a change from asparagine to aspartic acid at amino acid position 752 (N_{752} → D_{752}) are the strains with the strongest potential to cause neurologic disease (272, 452, 454). In chapter 2 of this dissertation, we hypothesized that EHV-1 strains vary in their ability to suppress IFN-β response and that this variation is associated with variation in viral neuropathogenicity. Our data revealed that all the studied isolates (both neuropathogenic and non-neuropathogenic based on D or N at 752) of EHV-1 had reduced induction kinetics of IFN-β mRNA at 12 hpi when compared to poly-I:C-treated controls (Fig. 2.2). No statistically significant correlations were observed between the IFN-β mRNA induced in infected EECs and neuropathogenic genotype of EHV-1 isolates. This data implied that the host cells’ release of IFN-β following EHV-1 infection is not determined by the presence of a mutation in the virus DNA polymerase. Indeed, both EHV-1 strains with the neuropathogenic genotype and non-neuropathogenic genotype have
viral factors mediating an inhibition of IFN-β response in host cells following infection. The classification of EHV-1 isolates into either neuropathogenic or non-neuropathogenic genotypes based on presence of a DNA\text{pol} SNP is overly simplistic as factors other than viral factors may influence neuropathogenicity (109). Our data refutes our hypothesis, and it is possible that both genotypes of EHV-1 possess similar pathological relevance within a host resulting in similar patterns of antigenic stimulation and IFN-β response observed \textit{in vitro}. This possibility is also supported by our data revealing that the presence of a DNA\text{pol} SNP in the neuropathogenic genotype of EHV-1, previously demonstrated to affect virus load \textit{in vivo} (411), exerts no influence in the extent of virus' cell-to-cell spread in EECs as measured by plaque assay. With influenza virus, defects in the viral gene (NS1) responsible for IFN suppression are correlated with reduced plaque size and attenuated replication in IFN-competent cells (529). It is, therefore, deducible from our study that the DNA polymerase of EHV-1 encoded by ORF30 is not the viral factor mediating type-I IFN shutdown in EECs during infection.

We next modified our hypothesis to investigate whether there is a correlation between pathogenicity of EHV subtypes and type-I IFN inhibition that we observed with EHV-1 field isolates. For this study, we compared the well characterized T953 strain of EHV-1 isolated from a previous neurologic outbreak with the T445 strain of EHV-4 which has reduced pathogenicity mainly confined to the URT. Data revealed that these two EHV subtypes produced different patterns of IFN-β induction in EECs with EHV-1 being a more potent inducer of
IFN-β mRNA early in infection (Fig. 2.3.A). This finding correlates with EHV-1 being more virulent than EHV-4 therefore, eliciting an immediate IFN response from infected host cell during infection. Interestingly, EHV-1 but not EHV-4 produced an antagonistic effect on type-I IFN production in EECs later in infection at 12 hpi. Consistent with our hypothesis, our data revealed that suppression of type-I IFN activity is a correlate of the pathogenicity of EHV subtype with the more pathogenic EHV-1 being able to inhibit type-I IFN in host cells. Although the magnitude and duration of viremia produced by EHV-1 have been described as critical factors enabling the delivery of EHV-1 to distant tissues (109), we opine that EHV-1 specifically shuts down host type-I IFN responses in endothelial cells of the pregnant uterus and CNS in order to aid virus cell-to-cell spread and replication. This eventually leads to the damage observed in the microvasculature of the small blood vessels around these secondary target organs leading to severe symptoms such as abortion and CNS disorders. We deduced that inhibition of type-I IFN is not a neuropathogenic phenomenon but rather a pathogenic attribute of EHV-1, irrespective of its genotype, that enhances virus spread and replication in host cells.

5.2.2 EHV-1 negatively regulates host factors required for type-I IFN production

Type-I IFN induction by virus-infected cells occurs in 3 phases—sensitization, induction, and amplification (302). Our findings together with others from our lab (31, 402, 406, 418) revealed that EHV-1 can inhibit type-I IFN responses, in the host cell, but there is still an open question of how the virus achieves this. We next explored the effect of EHV-1 infection on type-I IFN
signaling pathways with the aim of identifying the steps targeted for inhibition (chapter 3). At the beginning of the signaling events leading to type-I IFN production are a family of innate immune-recognition receptors called TLRs. TLRs recognize conserved molecular patterns (PAMPs) that are associated with microbial pathogens, and induce anti-microbial immune responses including type-I IFN (530, 531). In addition to stimulating TLR signaling events, other viruses (both RNA and DNA virus) have been demonstrated to inhibit TLR expression and signaling thereby, counteracting IFN production in the process (480, 532). We hypothesized that EHV-1 down-regulates the cellular expression levels of TLRs so as to dampen the innate immune response to infection. Our study demonstrated a statistically significant reduction of both TLR3 and TLR4 mRNA in EHV-1-infected EECs at 6 hpi and 12 hpi (Fig. 3.1). Downregulating the expression of TLR3 and TLR4 probably helps EHV-1 to escape being recognized as a pathogen favoring virus replication within host cells. Our study of TLR expression did not investigate the impact of EHV-1 on specific TLR signaling owing to poor performance of the TLR ligands we tested (TLR7 and TLR9 ligand). However, we believe that by inhibiting TLR3 and TLR4 expression, EHV-1 would have a negative effect on downstream signaling events which will hinder type-I IFN production. Although we did not test the hypothesis that EHV-1 similarly inhibits other cytosolic PRRs such as RLR, we speculate, based on our TLR data that EHV-1 utilizes same viral factors to evade cytosolic detection.

Among the nine members of the IRFs (IRF1-9), four have been associated with the transcriptional induction of IFN-α/β genes (533-535). Previously, our
laboratory reported that the T953 strain of EHV-1 inhibits host type-I IFN response by antagonizing IRF3 activation and subsequent nuclear translocation in EECs during infection (402). We next hypothesized that EHV-1 down-regulates IRF7 and IRF9 as part of its mechanism of IFN-α/β suppression. Our data supports this hypothesis (Fig. 3.2) indicating that EHV-1 specifically inhibits cellular levels of both IRF7 and IRF9 to block the induction of IFN-α/β genes during infection. Both IRF7 and IRF9 play distinct and essential roles which together ensure transcriptional efficiency and diversity of IFN-α/β genes for a robust antiviral response. While IRF3 and IRF7 form a complex-virus activated factor (VAF) that binds IFN-β promoter in the nucleus (536), IRF9 is a subunit of IFN-activated transcription factor termed 'ISGF3' which is involved in IFN-α/β induction (537-539). Inhibition of these important IFN transcription factors demonstrates a downstream effect of EHV-1 on efficient TLR signaling enabling the virus to avoid host antiviral response.

As described in the Introduction, IFN antiviral activity depends upon successful JAK-STAT signaling for induction of IFN-α/β. Some viruses including herpesviruses disrupt the JAK-STAT signaling pathways at different steps (404, 540, 541). Recently, our laboratory also demonstrated high resistance of EHV-1 to rEqIFNα-induced phosphorylation and nuclear translocation of STAT1 molecule in infected EECs (31). We hypothesized that EHV-1 has a similar effect on STAT2 during infection. Consistent with the previous result, our data showed that EHV-1 infection of EECs reduced phosphorylation of STAT2 and promoted cytosolic retention of STAT2 even in the presence of an exogenous IFN
stimulation (Fig. 3.4.A and Fig. 3.4.D). The possibilities are that either EHV-1 is directly preventing the nuclear accumulation of STAT2 by physically interacting with STAT2 or the virus is blocking the upstream activators of STAT2 molecule. We next hypothesized that EHV-1 blocks both STAT1 and STAT2 activation by interfering with the upstream kinases responsible for STAT1 and STAT2 phosphorylation. The Janus kinases, JAK1 and TYK2, are important upstream factors required for the phosphorylation-activation of STAT1 and STAT2 molecules (324, 325, 488). Our data revealed that while the cellular abundance of JAK1 remained constant with infection, EHV-1 infection degrades the cellular abundance of TYK2 at 12 hpi (Fig. 3.3). By down-regulating the cellular level of TYK2, we suggest that EHV-1 indirectly blocks the phosphorylation-activation of STAT1/STAT2 molecule. Other viruses such as human metapneumovirus have been shown to direct both JAK1 and TYK2 for proteosomal degradation, thereby, interfering with STAT1 and STAT2 phosphorylation (21). We are at this time unable to confirm whether an EHV-1 viral factor physically interacts with STAT2 molecule as is reported for HSV ICP27 (470). However, our data support the hypothesis that EHV-1 mediated inhibition of IFN-α/β induction comes by way of an ineffective JAK-STAT pathway. We also found that UV-inactivated EHV-1 is not able to block the phosphorylation of STAT1/STAT2 indicating that de novo viral gene expression or viral replication is required for reduced phosphorylation of STAT1/STAT2. Similarly, blocking the late gene expression by EHV-1 abolishes its suppressive effects on STAT1/STAT2 phosphorylation. The inhibitory effects of EHV-1 on IFN-α/β induction occurs at different levels of the
signaling cascade, as the virus blocks TLR3 and TLR4 gene transcription, inhibits IRF7 and IRF9 gene transcription, and facilitates TYK2 degradation, ultimately leading to inhibition of STAT2 activation. As a result, we speculate that the ability of EHV-1 to interfere with outside-in signal transduction and the JAK-STAT pathway will be consequential on the host type-I IFN response. Therefore, it is reasonable to hypothesize that EHV-1 encodes one or more viral proteins, presumably encoded by late genes, responsible for the sequential events targeted at achieving a reduced IFN-α/β induction in the infected host cell. Acquisition of these mechanisms for IFN evasion that can subdue multiple steps of IFN pathway may be one of the reasons why EHV-1 remains a successful pathogen of horses.

5.2.3 EHV-4 is unable to block host factors of type-I IFN production

In chapter 2, we showed that the ability to shut down the host type-I IFN response is not pathogenicity dependent among EHV-1 strains. EHV-1 and EHV-4 share 55 to 84 % nucleotide identity within the individual homolog genes and 55 to 96% homology exists between their amino acid sequences (77, 81). So, an EHV-1-coded IFN-suppressive factor might possibly be shared by EHV-4. In chapter 4, we investigated whether EHV-4 exerts similar effects as EHV-1 on type-I IFN induction steps during infection of EECs. EHV-4 replicates in EECs although endothelial cells are not its normal site of replication in the horse. EHV-4 failed to either induce or inhibit TLR3 and TLR4 mRNA even in the presence of a positive inducer (Fig. 4.1). These findings suggest that unlike EHV-1 which is more pathogenic, EHV-4 should not be able to evade recognition and oppose
downstream TLR signal transduction needed for IFN-α/β induction. Similarly, our
data showed that EHV-4 was unable to down-regulate the expression of both
IRF7 and IRF9 mRNA. Similarly, we do not expect EHV-4 to interfere with IFN-
α/β induction at any time during infection due to lack of inhibition of IRF7 and
IRF9 mRNA. Interestingly, EHV-4 suppressed the phosphorylation of
STAT1/STAT2 molecules but progressively lost this suppressive effect with time.
By blocking phosphorylation-activation of STAT molecules early during infection,
EHV-4 might prevent a robust IFN-α/β gene induction during early infection.
These data are in contrast to our EHV-1 data and may be related to the relative
inability of EHV-4 to replicate outside of the URT. Suppression of IFN-induced
STAT1/STAT2 phosphorylation is dependent on an active viral gene expression
by EHV-4. UV-inactivation of EHV-4 abolished these effects (Fig. 4.4).
Intriguingly, unlike EHV-1, viral late gene expression was not required for the
inhibition of STAT1 and STAT2 phosphorylation by EHV-4. PAA-mediated
blockage of EHV-4 late gene did not abolish the suppressive effect on STAT1
and STAT2 phosphorylation at 3 hpi and 6 hpi (Fig 4.5.A, Fig. 4.5.B, Fig. 4.5.D,
and Fig 4.5.E). We speculate that EHV-1 and EHV-4 utilize different viral genes
or pathways for interfering with STAT1 and STAT2 phosphorylation. It would be
interesting to find out the effect an EHV-1-EHV-4 chimera, expressing only EHV-
4 late genes, would have on type-I IFN in vitro. We predict a reduced IFN-
suppressive effect from such chimera in infected EECs. Individual field isolates of
EHV-1 and EHV-4 exhibit wide variations in virulence in horses (206, 213).
Hypervirulent strains of EHV-1, such as the ones in this study, are highly
endotheliotropic causing high rates of abortion and neurologic syndromes (213, 229). However, EHV-4 primarily causes respiratory disease and normally does not lead to abortion and neurologic disease due to reduced endothelial cell replication and cell-associated viremia (77). Our data show that EHV-1 but not EHV-4 has the inherent ability to shut down the host IFN-α/β response in infected cells. This exerts a major influence on the diverse replication and invasion strategies exhibited by the two EHV subtypes. In vivo, we therefore expect EHV-1 to be able to avoid triggering an immediate IFN-α/β alert and also be able to inhibit IFN-α/β response induced by other co-infecting unrelated pathogens enabling the virus to promote secondary pathologies. This provides further explanation of the differential pathogenic potentials that exist between EHV-1 and EHV-4 during natural outbreaks of infection.

5.3 Limitations

One of the caveats of our EEC model employed for this project is a direct extrapolation of our novel findings to reflect natural events in an intact animal model or a horse. The complexity in the architecture and organization of different organs within a horse may influence the outlook of our study were it conducted in vivo. In an intact horse, a robust induction of the full complement of the host immune response may have an overlapping role in moderating EHV-1 disease establishment which may make an exaggeration of our in vitro results. Although we were able to establish EHV-1 and EHV-4 infections in EECs, direct infection of the endothelial cells with free virus particle may not be reflective of a natural infection in field outbreaks. Both EHV-1 and EHV-4 are primarily respiratory
pathogens with tropism for equine respiratory mucosal epithelium that permits efficient viral entry into the horse (77), and indeed, most respiratory pathogens are disposed of by the mucociliary escalator even if they evade nasal filtering and end up in the trachea (542). Therefore, care must be taken in interpreting our results as it may be seemingly unlikely for free virus particles, in high titers, to have direct access to the endothelium of different tissues after an initial escape from the URT in a healthy horse.

The lack of expression of certain TLRs in EEC also places limitations on the scope of this dissertation. Although thirteen mammalian TLRs have been recognized, only TLRs 2-4 and 6-9 have been found to be expressed in equine cells (543-547). While TLR7 recognizes viral ssRNA, TLR9 detects non-methylated viral CpG-containing DNA motifs in dsDNA viruses (548). Our EEC model failed to show induction of the expression of TLR7 and TLR9 even when stimulated with the corresponding TLR7 (tlrl-gdq-5) and TLR9 (tlrl-dsl03) agonists (Invivogen, CA). While other researchers have demonstrated the expression of these two genes in different horse studies (547, 549), it is possible that their expression is cell-type specific. A few other studies have also reported low level expression or lack of expression of both TLR7 and TLR9 mRNA in equine and human epithelium tissue samples (550, 551). Our results suggest that both TLR7 and TLR9 are not required for sensing EHV-1 genetic material in EECs. The overlapping nature of the TLR signaling pathway may help compensate for any lack of TLR7 and TLR9 expression in EECs ensuring that viral pathogens do not escape detection.
Another limitation of this study is our inability to evaluate the posttranslational cellular abundance of TLR3, TLR4, IRF7, and IRF9 proteins following EHV-1 infection of EECs. Evaluating the biological effects of EHV-1 on host gene expression without a protein data may present an incomplete description of the mechanism being studied. It is possible that certain posttranslational modifications occur following gene transcription during EHV-infection of EECs which may directly influence the global picture of our data were protein data available. During the course of this study, we were confronted at one point or the other with either lack of availability of an equine-specific antibody reagent, or ineffectiveness of the available equine-specific antibody. This limited the scope of our study and restrained us from asking other fundamental questions that would have provided additional details to the current findings. Future work should focus on delineating the impact of EHV-1 on protein products of TLR3, TLR4, IRF7, and IRF7 mRNA following infection. When useful equine-specific antibodies are available, it will also be fascinating to investigate whether EHV-1 targets TLR-mediated recruitments of adaptor proteins during infection.

5.4 Future directions

This dissertation has provided valuable insights into the previously unknown mechanisms employed by EHV-1 to circumvent type-I IFN responses in host cell. It is our hope that this work encourages future exploration into the lifecycle of EHV-1 and host-pathogen interactions affecting the quality of innate immunity, particularly the type-I IFN response. Perhaps one of the crucial unanswered questions of this project is identifying the exact viral factor utilized by EHV-1 to
inhibit the host type-I IFN response. Throughout the course of our study, EHV-1-mediated inhibition of host factors (e.g. STAT, TYK2) required for type-I IFN response occurred predominantly at 12 hpi. Coincidently, the observed timing corresponds to when EHV-1 significantly down-regulates type-I IFN in host cells (Fig. 2.3.B). This implies that the viral factor responsible for type-I IFN inhibition in the host cell is expressed by 12 h during the virus lytic life cycle. In chapter 3, we also presented data that supports our hypothesis that the viral factors responsible for anti-IFN response in host cells are late-expressed factors (Fig. 3.7.C). Similarly, a previous study had also reported the involvement of a late protein of pseudorabies virus, another alphaherpesvirus, in the blockage of IFN-mediated phosphorylation of STAT1 (552). We, therefore, propose that the following selected late genes of EHV-1 be screened for their inhibitory effect on type-I IFN response in host cells. Studies on HSV-1 have revealed that important viral proteins, such as ICP34.5, US11, ICP0, ICP27, UL13, and UL41, target different steps of the type-I IFN system including production of IFN, IFN signaling, and the functioning of ISG products (470, 524, 553-556). While EHV-1 encodes functional homologs of HSV-1 ICP0, ICP27, UL13, and UL41 proteins, it lacks both ICP34.5 and US11 (Table 1.2). Future work in our laboratory will seek to characterize the functional relevance of EHV-1 ORF 63 (HSV-1 ICP0 homolog), EHV-1 ORF5 (HSV-1 ICP27 homolog), EHV-1 ORF49 (HSV-1 UL13 homolog), and EHV-1 ORF19 (HSV-1 UL41 homolog), on type-I IFN pathway in equine cells. This might be achieved by evaluating the impact of infectious clones of EHV-1, containing mutations of reported IFN-suppressing HSV-1 homologs,
on type-I IFN response in infected EECs using reporter assays. We also hope to screen selected EHV-4 IE and E genes that are possibly functional homologs of documented HSV-1 genes involved in STAT1/STAT2 phosphorylation-inhibition.

The advent of next generation sequencing (NGS) has revolutionized our abilities to formulate scientific questions and advance knowledge. NGS has predominantly been used in studying infectious pathogens such as HIV (557-559) and hepatitis B virus (560-562) but it is now gaining traction for other viruses including HSV-1 (563-565). In the future, we propose to conduct a comprehensive transcriptome profiling of the host type-I IFN response to EHV-1 through NGS. The first approach of this study would be accomplished in vitro in EHV-1 target cells such as equine RECs and EECs. A second approach would be to directly address the host mediated anti-EHV-1 innate immune responses in vivo, using an experimentally infected horse model. Using both in vitro and in vivo approaches would provide a better picture of what is going on in arm races between EHV-1 and its host as infection progresses. We predict that using this approach would reveal key host innate immune transcriptomes that are either downregulated or upregulated during EHV-1 infection. This could potentially be used to identify host factors that contributes to the development of EHM. Similarly, modern technologies such as generation of an infectious EHV-1 clone system and CRISPR-Cas9 can be used for making many mutations in a short form to tease out EHV-1 protein functions and host response once a specific viral gene target has been identified.
Another fascinating area of future research is to delineate the role of the respiratory microbiome during EHV-1 infection. There is now a global shift of how animal and human disease conditions are managed in terms of minimizing therapeutic interventions while at the same time obtaining an optimal result. A study on the alterations in the upper respiratory tract biota in EHV-1-infected horses can provide a viable approach to managing the initial stage of the respiratory disease. Since EHV-1 gains entry and first replicates in the respiratory epithelial cells, we would like to study how EHV-1 affects the diversity of the respiratory tract microbiome in infected horses. The phenotypes of the upper respiratory tract microbiome from EHV-1 infected horses could be compared with those from healthy control horses using NGS. Recent reports have indicated that the gastrointestinal microbiota exerts an essential role in maintaining intestinal epithelial cell integrity and development of the mucosal immune system (566-570). We propose that there would be a reduced diversity of the respiratory tract microbiome in EHV-1-infected horses when compared to healthy uninfected controls. This study would reveal the identity and possible roles of missing microflora in the respiratory tract of the EHV-1-infected horses. Restoration of the respiratory tract flora population by microbiota transplant can potentially help improve the health of EHV-1-infected horses by reviving the lost mucosal immunity during infection. The major advantage of this approach is that it removes the risk of adverse effects of administering medicine or that of vaccination. This approach has been tried with great success for managing
gastrointestinal diseases and can also be adapted as a potentially viable approach for managing the initial respiratory disease associated with EHV-1.

Other compelling questions, with accompanying speculation, include:

1. Does EHV-1 equally antagonize type-III IFN?

Three classes of IFN, Type-I, II and III, have been characterized (571). Functionally, type-III IFN (IFN-λ) produces effects very similar to IFN-α. These include induction of antiviral, anti-proliferative, anti-tumor, and other innate immune responses (572). Although the IFN-λ receptor complex is unique, once activated by ligand binding it signals through the same intracellular pathways as IFN-α/β which accounts for its producing much the same functional effects including antiviral activity (571, 573, 574). Our current data revealed that EHV-1 is equipped with viral factors which mediate an anti-type-I IFN response in EECs. By extension, similar questions can be asked about the impact of EHV-1 on IFN-λ response at the mucosal epithelium of the URT. This will offer a supportive detail on how EHV-1 is able to manifest and establish infection in its initial primary replication site. We predict that EHV-1 will block type-I IFN responses as well, by using the same mechanisms as for type-I IFN.

2. Does EHV-1 inhibits type-I IFN response in uninfected bystander cells?

In chapter 2, we demonstrated that inhibition of type-I IFN response in host cells is a pathogenic property of all EHV-1 pathotypes. The ability to evade the type-I IFN response of the host facilitates virus cell-to-cell spread during EHV-1 infection. This raises another question: is EHV-1-mediated anti-IFN response restricted to only virus-infected cells? In the future, we intend to carry out single
cell studies that will look at host responses to EHV-1 effects in both virus-infected cell and virus-uninfected cell populations. This would provide a global picture of the extent of IFN suppressive effect that is present during EHV-1 infection. If EHV-1 did suppress IFN response in uninfected cells, we would be interested in delineating the steps in the type-I IFN system pathways that are likely targeted as a result. We predict that EHV-1-mediated IFN suppression effect does not affect neighboring bystander cells.

3. What host factors contribute to the development of EHM?

The factors that can be directly linked to the development of EHM in horses are ill defined. While some studies have attributed this phenomenon to the presence of an SNP in the ORF30 encoding DNA polymerase in EHV-1 (272, 452), there is still some debates about this explanation. Furthermore, our data suggest that both EHV-1 with the neuropathogenic genotype and EHV-1 strains with the non-neuropathogenic genotype behave similarly in vitro in affecting the type-I IFN response. It has also been reported that older horses are more susceptible to the development of EHM (411, 575), suggesting that, possibly, host factors may be responsible for the development of this phenotype. It is also noteworthy that only the endothelial cells of some selected organs such as conjunctiva, pregnant uterus, and CNS are EHV-1-affected. Future studies may characterize the cell surface adhesion molecules or markers on these EHV-1 target organs relative to other organs to answer if, indeed, there are certain host determinants of EHM.
5.5 Conclusions

Here, we gathered evidence in support of our hypothesis that pathogenic strains of EHV-1 encode viral factors that allow them to overcome the potent antiviral effects of the host type-I IFN response during infection. During natural outbreaks in horses, EHV-1 particularly targets endothelial cells of important organs such as the respiratory tract, the conjunctiva, pregnant uterus, and the CNS. Our data suggest that suppression of the type-I IFN response in host cells is among the first important steps needed by virulent strains of EHV-1 to produce diseases in vivo. The data herein provide compelling evidence identifying some of the underlying, novel, molecular pathways targeted by EHV-1 in order to down-regulate host type-I IFN response during infection. The inability of EHV-4 to inhibit type-I IFN responses in host cells may partly explain why the virus rarely produces fatal outcomes such as abortion and neurologic diseases. These data support our central hypothesis and provides valuable insights into how pathogenic EHV-1 manipulate the type-I IFN signaling pathway in order to promote disease (Fig. 5.1). Our data has provided the groundwork for the identification of therapeutic target(s) of EHV-1. Further investigation to identify the viral protein(s) utilized by EHV-1 to resist host type-I IFN response may help fuel the discovery of novel therapeutic that will be effective against EHV-1 disease outbreaks.
Figure 5. Schematic illustration of our proposal of how EHV-1 T953 strain blocks the key molecules required for type-I IFN production. (A) During the sensitization phase of host type-I IFN production, EHV-1 blocks the expression of TLR3 and TLR4 mRNA thereby enabling the virus to avoid host detection. (B) In the subsequent induction phase, EHV-1 specifically degrades the cellular level of TYK2 protein. This consequently blocks the abundance of cellular levels of phosphorylated/activated STAT1 and STAT2 molecules in infected cells. At the same time, EHV-1 blocks the transcription of IRF9 mRNA and thus prevents the formation of ISGF3 complex needed to transactivate ISRE for ISG production. EHV-1 also inhibits the transcription of IRF7 mRNA, exerting a negative effect on IFN-α production. The overall effect of EHV-1 renders the host cell unable to restrict viral spread by direct cell-to-cell contact during infection.
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