MODULATION OF TYPE-I INTERFERON MEDIATED IMMUNE RESPONSE: A NOVEL INNATE IMMUNE EVASION STRATEGY OF EQUINE HERPESVIRUS 1

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MODULATION OF TYPE-I INTERFERON MEDIATED IMMUNE RESPONSE: A NOVEL INNATE IMMUNE EVASION STRATEGY OF EQUINE HERPESVIRUS 1

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky
By
Sanjay Sarkar
Lexington, Kentucky

Director: Dr. Thomas M. Chambers, Associate Professor, Department of Veterinary Science
Lexington, Kentucky
2014

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

Modulation of Type-I Interferon Mediated Immune Response: A Novel Innate Immune Evasion Strategy of Equine Herpesvirus 1

Equine herpesvirus-1 (EHV-1) is one of the major viral pathogens causing respiratory disease, abortion, perinatal mortality and neurologic disease among horses resulting in significant economic losses to the equine industry. The virus can also remain latent in the horses and recrudesce at any time. Type-I interferons (IFNs) act as a first line of defense against many viral infections.

In this study we investigated the type-I IFN response against the neuropathogenic T953 strain of EHV-1 in equine endothelial cells (EECs). The results showed that after a transient induction of IFN-β mRNA as well as protein at an early time (3h) post infection (p.i.), T953 strain of EHV-1 suppressed further induction of IFN-β at later times (12h onwards). Studies were done to confirm that the suppression of type-I IFN induction at later time points was not due to the normal IFN-β induction kinetics, it was rather because of the active interference by the virus. Investigation of the mechanisms by which T953 interferes with IFN-β production revealed that the virus degraded the endogenous level of the transcription factor, interferon regulatory factor 3 (IRF-3) and also down-regulated the activation of IRF-3 followed by its accumulation in the nucleus. However, T953 infection caused degradation of nuclear factor κB (NF-κB) inhibitory protein IκBα and also induced p50 subunit to translocate into nucleus from cytoplasm suggesting activation of NF-κB signaling. This also indicated that inhibition in the type-I IFN production was probably not due to the inhibition of NF-κB.

The results of these studies also indicated that T953 virus was resistant to the biological effect of the recombinant equine IFN-α in vitro. Investigation of the reason of this resistance showed that T953 virus interfered with the cellular JAK-STAT signaling mechanism by which type-I IFN exerts its antiviral effect. Moreover, the studies revealed that downstream of the JAK-STAT signaling, T953 virus also inhibited the expression of cellular antiviral proteins including interferon stimulated gene 56 (ISG56) and viperin. Altogether, these data indicate that the T953 strain of EHV-1 interfered with the host cell innate immune responses by modulating type-I IFN mediated immune responses at multiple levels in vitro.

KEYWORDS: Equine herpesvirus-1, Interferon, Interferon regulatory factor-3, JAK-STAT signaling, Interferon stimulated gene

Sanjay Sarkar

January 14, 2014
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DEDICATION

I want to dedicate this dissertation to the most amazing person in this world I’ve ever met, Mrs. Usha Bhowmik. Without her care and love, support, suggestions I would not be able to become what I am today. I love you Maa! Thank you so much for everything.
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CHAPTER I: REVIEW OF LITERATURE

1.1 Introduction and History of Herpes:

The word ‘herpes’ was originally derived from the Greek word *herpein* meaning to creep or crawl because of the creeping nature of the lesions on the skin caused by the viruses. The origin of the word ‘herpes’ probably dates back more than twenty five centuries although the first written documentation of human herpes virus infection is believed to be done by Hippocrates (460 BC-377 BC) [1-3]. Similar signs of infections have also been mentioned in many other ancient documents, e.g., the Sumerian Tablet (3rd Millenium BC), Egyptian Ebers Papyrus (circa 1500 BC) or in Avicenna’s documentation (880-1036 AD). Celsus was the first who described the actual herpetic lesion as ‘initially round but then diffused like serpent’. These signs were caused by the herpesvirus now known as varicella zoster virus (VZV) which was also documented by Herodotus and later elaborated by Galen [2].

Over the centuries the term ‘herpes’ and its meaning have been changed several times to ‘formica’, furfur and again back to ‘herpes’. In early medical history the word ‘herpes’ was attributed to many pathological conditions of the skin like skin cancer, noma, erysipelas, and ringworm including lupus vulgaris [2, 3]. The Byzantine author, Actuarius (John Zachary) is considered to be the first author to certainly use the term ‘herpes’ for cutaneous ringworm (reviewed in [3]). In 1275, Saliceto considered ‘herpes’ to be different from ‘erysipelas’ as the latter only involves the skin while ‘herpes’ involves also underlying tissues (reviewed in [3]). Gorraeus recognized 3 different types of herpes i.e., ‘H. esthiomenos’, ‘H. miliaris’ and ‘H.phlyctenodes’ [4]. In contrast to Saliceto, Daniel Sennert still believed that ‘herpes’ and ‘erysipelas’ both were caused by same agents and he also mentioned 3 different types of herpes in his writings, such as ‘H. simplex’ (‘Herpes vero solam cutem exulcerat’), ‘H. esthomenos’ or ‘H. exedens’ and ‘H. miliaris’ [5]. Daniel Turner, who gave more modern descriptions compared to these previous authors, considered ‘herpes’ to be ‘a choleric pustule’[6]. He is the one who described herpes simplex under its modern name [3].
pustule’[6]. He is the one who described herpes simplex under its modern name [3]. In the beginning of the nineteenth century Robert Willian and Thoms Bateman contributed significantly to the field of herpes virology. Bateman recorded 6 different species of ‘herpes’ which are *H. phlyctenodes, H. zoster, H. circinatus, H. labialis, H. praeputialis and H. iris* [3]. Unna in 1883 elaborately described the recurrence of herpes infection [7]. Three years later in 1886 a French dermatologist, Jean Baptiste Emile Vidal, first showed that skin conditions caused by herpes simplex were infectious and could be transmitted from human to human [2, 8]. The great discovery of the first virus, tobacco mosaic virus and the fact that agents different and smaller than bacteria can cause disease by Dmitri Ivanovski and Martinus Biejerinck in 1892 and 1898, respectively, set the milestone for virology research [9, 10]. Within the same time period the first book dedicated to herpes, ‘*Les Herpes Genitaux*’ was published in 1896 by the French doctors Charles-Paul Diday and Adrien Doyon [11].

While herpesviruses have been considered to be evolving along with their hosts over a period of about 400 million years, the subgroup *alpha* herpesviruses have diverged only 180-210 million years ago [12, 13]. Although human herpesvirus infections started a long time ago, it was not until the past four centuries that clinical findings of equine herpes virus were recorded [14].

### 1.2 Importance of EHV-1 Infection to United States Horse Industry

Kentucky is considered to be the horse capital of the United States (US). The US equine industry is a very diverse industry involving almost all the states in US providing an opportunity for at least 460,000 direct full-time employment jobs (FTE). 4.6 million Americans of which 2 million are horse owners are directly involved, and many others are indirectly involved with this industry, making every 1 out of 63 Americans involved with horses [15]. The industry involves agriculture, business, and recreation impacting significantly at national, state and local economies. The 102 billion US dollar (USD) equine industry consists of racing (26.1 billion), showing (28.7 billion), recreation (31.9 billion), other (14.6 billion) USD (Figure 1.1) and pays
revenue of 1.9 billion to the US government which includes Federal (588 million), State (1017) million and local (275 million) USD revenues [15].

![Distribution of the economic sectors of the US horse industry](image)

**Figure 1.1: Distribution of economic sectors of the US horse industry.** Of the 102 billion USD horse industry, the most significant contribution comes from recreation followed by showing, racing and others. The figure is made from the information available from American Horse Council Foundation [15].

Out of the 9.2 million horses in the US, Kentucky has about 320,200 horses and 194,300 Kentuckians are involved in the industry either as horse owners, service providers, employees or volunteers [15]. The equine industry in Kentucky creates direct 51,900 FTE with a total impact of 96,000 job opportunities. The horse industry in Kentucky has an impact of 3.54 billion USD in terms of gross domestic product added directly to US economy.

EHV-1 infection causes serious damage to the horse industry worldwide including in the US. As a direct effect of the infection, horses suffer from respiratory disease, neurologic disorders, abortions, and neonatal death. The infection also makes horses susceptible to other secondary bacterial infections like *Streptococcus equi subsp. zooepidemicus* [16]. Even though
horses may recover from the disease eventually, their productivity is compromised. Late term abortions usually in the last trimester and neonatal death cause significant economic losses to horse breeders and also set back the industry as whole. EHV-1 induced neurologic disease, also known as equine herpes myeloencephalopathy (EHM) causes significant morbidity as well as mortality. The restriction in the movement of horses nationally and internationally caused by EHV-1 infection and the rescheduling of horse shows and races also impact the equine industry negatively. Large amount of money are also spent on the care and management of horses infected with EHV-1.

1.3 Classification of Herpesviruses:

The first organized classification of herpesviruses came in 1971 by the International Committee on Taxonomy of Viruses (ICTV) [17] and the most recently updated classification was adopted in 2009 by the ICTV [18]. Here the newly formed order Herpesvirales are considered to have three distinct families: the Alloherpesviridae, the Malacoherpesviridae and the Herpesviridae, the latter of which is also the largest amongst the groups. While the Alloherpesviridae includes herpesviruses of fish and frogs and the Malacoherpesviridae includes a bivalve virus, the revised family of Herpesviridae includes herpesviruses of mammals, reptiles and birds [18]. The classifications of different herpesviruses into different families are largely based on virion morphological criteria [19].

The members of the herpesviridae family have spherical virion morphology with four major components: the core, the capsid, the tegument and the envelope (Figure 1.2). The core contains a single copy of a linear, double-stranded deoxyribonucleic acid (DNA) molecule which is arranged with unique long and unique short regions with terminal and internal repeats. The core DNA molecule is encased within an icosahedral capsid with an external diameter of 125-130 nm [18, 19]. The capsid is made of 162 capsomeres of which 12 are pentons and 150 are hexons [19]. This is surrounded by unstructured proteinaceous matrix, the tegument which consists of 30 or
more viral proteins [18]. The external lipid bilayer membrane, or envelope, surrounds the tegument and a number of glycoproteins of both virus and host origin are embedded into the envelope [19].

![Diagram of herpesvirus virion]

**Figure 1.2: Basic Architecture of Herpesvirus Virion.** All herpesviruses have four major components, the core which consists of a dsDNA genome, capsid, tegument and envelope. Several glycoproteins are embedded in the envelope.

Based on the biological criteria, the members of the herpesviridae family are categorized into three subfamilies: *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae* [20]. These subfamilies are further assigned into different genera based on molecular data; mainly DNA sequence homology, genome size and structure and relatedness of viral proteins [14, 21].
The members of the *Alphaherpesvirinae* subfamily are distinct from the other subfamilies by their characteristic variable and wide host range, relatively short replication cycle (few hours), rapid spread in cell culture with cytolysis and the capability to establish life-long latency in the sensory ganglia of the central nervous system or in the lymphocytes of their hosts [22]. The subfamily contains four different genera: *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus* [19]. Members of the *Simplexvirus* include Human herpesvirus-1, Human herpesvirus-2, Bovine herpesvirus-2, Ateline herpesvirus-1 and Cercopithecine herpesvirus-1 [19]. Members of the *Varicellovirus* include Bovine herpesvirus-1, Bovine herpesvirus-5, Suid herpesvirus-1, Equid herpesvirus type-1, -3, -4, -6, -8 and -9, Caprine herpesvirus-1, Canid herpesvirus-1 and Human herpesvirus-3 [19]. Members of the *Mardivirus* infect only birds and include Gallid herpesvirus-2 (Marek’s disease virus type-1), Gallid herpesvirus-3 (Marek’s disease virus type-2) and Meleagrid herpesvirus-1 (Turkey herpesvirus) [18, 19]. Members of the Iltovirus include Gallid herpesvirus-1 (Infectious laryngotracheitis virus) and Psittacid herpesvirus-1 (Pacheco’s disease virus) [18].

The characteristic features of the members of the *Betaherpesvirinae* subfamily include restricted host range, long reproductive cycle (lasts several days), slow growth in cell culture and a characteristic cytomegalia of the infected cells with intranuclear inclusion bodies [14, 22]. In many cases the virus causes no apparent clinical symptoms and can be latent in leucocytes, cells of reticuloendothelial systems, salivary glands, kidneys and other tissues [23]. The subfamily consists of three genera: *Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus* [24]. Cytomegaloviruses have large genome size (>200 kbp) and cause characteristic enlargement of the infected host cells [24]. Examples of this genus include Human cytomegalovirus and African green monkey cytomegalovirus [24]. Muromegaloviruses which include two members, mouse and rat cytomegalovirus, also have large genome size (>200 kbp). Roseoloviruses, on the other
hand, have smaller (<200 kbp) genome size and infect primarily T cells and the two members of this genus include Human herpesvirus-6 and Human herpesvirus-7 [24].

The members of the subfamily Gammaherpesvirinae infect T or B lymphocytes and have variable replication cycle [22]. The gammaherpesviruses are very restricted in their host range (restricted to the taxonomic family or order of their natural host) and are often found latent in the lymphoid tissues [14]. This subfamily consists of four genera: Lymphocryptovirus, Rhadinovirus, Macavirus and Percavirus [18]. The members of Lymphocryptovirus include the Epstein-Barr virus and Gorilla herpesvirus and these viruses infect only primates [24]. The members of the Rhadinovirus have more varied host range compared to other genera of this subfamily and include Bovine herpesvirus-4, Macacine herpesvirus-5 and Human herpesvirus-8 [18]. The members of the Macavirus genus are genetically very similar and include Caprine herpesvirus-2, Alcelaphine herpesvirus-1 and Suid herpesvirus-3 [18]. Members of Percavirus include Equine herpesvirus-2, Equine herpesvirus-5 and Mustelid herpesvirus-1 [18].

1.4 Herpesvirus Infections of Equids:

The equid herpesviruses are one of the most prevalent viral pathogens found among equids causing a number of diseases. Equid herpesviruses belong to the order herpesvirales and family herpesviridae but to date all the equid herpesviruses isolated are restricted to either alphaherpesvirinae or gammaherpesvirinae subfamily. The members of the subfamily of alphaherpesviruses of equids include equid herpesvirus-1 (EHV-1), equid herpesvirus-3 (EHV-3), equid herpesvirus-4 (EHV-4), asinine herpesvirus-1 (AHV-1), asinine herpesvirus-3 (AHV-3) and Gazelle herpesvirus-1 (GHV-1) and all of these viruses belong to Varicellovirus [19]. The members of the gammaherpesviruses of equids include equine herpesvirus-2 (EHV-2), equine herpesvirus-5 (EHV-5) and asinine herpesvirus-2 (AHV-2). The most common equine herpesviruses that infects a majority of world’s domestic horses (Equus caballus) are EHV-1 and
EHV-4 that have been found for more than 70 years to be significant impediments to the success of breeding, competition and recreational horse industries [25].

1.4.1 Equine Herpesvirus Infections:

1.4.1.1 EHV-1 Infection:
EHV-1 infection causes respiratory disease characterized by fever (39.5-41.5 °C), malaise, inappetence, serous nasal discharge, pharyngitis, coughing and submandibular or retropharyngeal lymphadenopathy, neonatal death, ocular disease, epizootic abortion storms mainly in the late trimester as well as sporadic abortions, and also a neurological disorder known as EHM [26]. Progressive secondary bacterial infection may lead to rhinopneumonitis [27]. Although horses of any age group or breed or sex are susceptible to the viral infection, young horses are more susceptible to the respiratory form of the disease and the older horses are more susceptible to the development of EHM. Beside domestic horses, the virus has also been isolated from onagers (Equus hemionus onager), zebras and gazelles (Gazella thomsoni) [28]. The virus can be latent in lymphocytes (mainly CD8+ T lymphocytes) and also in sensory nerve cell bodies within the trigeminal ganglia of the infected horse which then becomes a carrier for its lifetime [25].

1.4.1.2 EHV-2 Infection:
EHV-2 along with EHV-5 was originally described as ‘slowly cytopathic orphan’ herpesvirus of domestic horses and was also classified under cytomegalovirus like betaherpesviruses [29-31]. With the availability of advanced biotechnological tools, complete genomes of both the viruses have been sequenced and the data led EHV-2 and EHV-5 to be incorporated under the subfamily of Gammaherpesvirinae and genus Percavirus [29, 32, 33]. Initially, the EHV-2 strain isolated in 1962 was a prototype LK strain from a foal suffering from ‘catarrh and coughing’ by Plummer and Waterson [34] and after that the virus has been isolated in different parts of the globe [29, 35, 36]. The virus has a linear dsDNA of 184 kbp length and 57% G+C content genome which encodes 77 distinct proteins [29, 32]. Foals of young ages are
more susceptible to EHV-2 infection [30, 37]. The virus can be shed persistently, intermittently or continuously or even be latent with sporadic reactivation and this could be very significant epidemiologically. It has been noticed that EHV-2 can be shed intermittently from the mucous membranes of the respiratory tract of horses for up to 14 weeks [38, 39]. EHV-2 has been isolated from peripheral blood leukocytes of 89% of the horses tested in the US and 90% in England [29, 40]. Moreover, the same animal can be infected with EHV-2 and EHV-5 at the same time [41]. Although endemic in the US, severe outbreaks of EHV-2 infections associated with respiratory diseases have also been reported [42]. In the field conditions, horizontal transmission of the virus is the most common route of spread to new animals [30]. Nasal mucosa, histiocytes and Langerhans cells in conjunctiva, and peripheral blood lymphocytes have been shown to be reservoirs and sources of excretion of EHV-2 in the environment [43-45]. Severe EHV-2 infections can show 100% morbidity with the symptoms of respiratory disease like increase in body temperature (38.5-39.5 °C), dullness, malaise, enlarged lymph nodes, chronic pharyngitis, coughing, serous to mucopurulent nasal discharge for several days and also keratoconjunctivitis [42, 46-49]. EHV-2 has also been linked to the poor performance syndrome in young performing horses and also to immunosuppression of the affected horses [49, 50]. In some isolated cases, EHV-2 has also been associated with naturally occurring abortions of mares as well as granulomatous dermatitis [51-53].

1.4.1.3 EHV-3 Infection:

EHV-3 is the causative agent of an infectious, mucocutaneous, venereal disease of mares and stallions known as equine coital exanthema (ECE) which is characterized by the formation of pock-like eruptions and erosions or ulcers on the external genitalia of mares and stallions [54, 55]. Although the virus is highly contagious, it does not normally cause any abortion or infertility or any systemic illness [54, 56]. The virus is antigenically, genetically and pathogenetically different from any other alpha or gamma equine herpesviruses and bearing only 2 to 5 per cent homology with EHV-1 at DNA level [57]. Unlike EHV-1, EHV-3 has a restricted host range and
grows in vitro only in the cells from equids. The virus grows optimally at 34 °C and an increase in temperature up to 39 °C could reduce the infectious progeny virus 10^6 fold [56, 58, 59]. Latently infected horses act as biological reservoirs and can shed the virus periodically, serving as a potential source of infection for other animals within the population [54]. It has been estimated that about 18-53% population of horses of breeding age possess EHV-3 specific antibodies [54, 60, 61]. Young and unbred horses are less susceptible to EHV-3 compared to older and breeding horses [60, 61]. EHV-3 is principally transmitted by direct skin-to-skin contact during coitus from an actively infected virus shedding horse but non-coital transmission by contact with virus contaminated buckets, instruments, gloves, or examination sleeves used for the purpose of breeding is also seen [54, 55]. After entry into a new horse, the replication of the virus is limited within the stratified epithelium of the epidermis or mucocutaneous junctions of the nares, urethral orifice, or female genital vestibule; and systemic dissemination of the virus has not been seen [54]. Both virus-neutralizing (VN) antibodies and complement-fixing (CF) antibodies are detected in sera from EHV-3 infected horses where VN antibodies persist longer [61]. No commercial vaccines are available which prevent ECE [54].

1.4.1.4 EHV-4 Infection:
After EHV-1, the most significant and ubiquitous alphaherpesvirus of equids is EHV-4 [25]. Unlike EHV-1, EHV-4 infection causes primarily respiratory disease and normally does not lead to EHM. However, sporadic, single cases of abortion have been documented occasionally after EHV-4 infection in horses [25, 37]. Although EHV-1 and EHV-4 are two distinct viruses, they share a great deal of antigenic and genetic similarities. EHV-4 has a linear double stranded DNA genome of 145.6 kbp with 50.5% GC content [25]. EHV-1 and EHV-4 share 55 to 84 per cent nucleotide identity within the individual homologue genes and the degree of homology in amino acid sequences ranges from 55 to 96 per cent [25, 62]. Because of the high degree of homology between two viruses, they were considered to be different subtypes of EHV-1 (subtype-1 or S-1
for EHV-1, subtype-2 or S-2 for EHV-4) until 1981 [63]. After that the DNA sequencing of EHV-1 and EHV-4 confirmed the individual identity of the two viruses [62, 64].

1.4.1.5 EHV-5 Infection:
Along with EHV-2, EHV-5 is another widely distributed gammaherpesvirus of equines [29]. The virus was originally isolated from the nasal cavity of two horses from the UK in quarantine in Australia in 1970 [42, 65] and since then EHV-5 had also been isolated from the peripheral blood lymphocytes (PBL) of horses suffering from upper respiratory tract diseases or also from apparently healthy horses in the US, Australia and Europe [32, 41, 66, 67]. EHV-5 has a linear dsDNA genome of 179 kbp length and a G+C content of 52% [68]. Though the virus shares an overall 60% homology with EHV-2 at the amino acid level, the two viruses are distinct entities [29, 32]. Like EHV-2, EHV-5 has also been shown to exhibit a broad host range and a longer replication cycle \textit{in vitro} in cell culture [29, 69]. In the majority of the cases, EHV-5 infected horses have shown inapparent or minimal signs. But recently, EHV-5 has been associated with the development of characteristic equine multinodular pulmonary fibrosis (EMPF) and also to secondary bacterial infection caused by \textit{Rhodococcus equi} [42, 70, 71]. Williams et al have described the progressive nodular fibrotic lung disease caused by EHV-5 and showed that the lungs of the infected horses contained multiple nodules with marked interstitial fibrosis [70]. Histological studies found that the airways of the affected horses contained neutrophils and macrophages containing the inclusion bodies which were confirmed to be ‘herpesviral-like particles’ by transmission electron microscopy [70]. In another report, Wong et al have also described EMPF in five horses with multifocal coalescing nodules of fibrosis in the lung parenchyma [72]. In other studies Schwarz et al [73] also reported similar EMPF cases in five horses in Hungary and Spelta et al [74] documented EMPF cases in three horses in Australia. In a very recent study Williams et al infected horses with EHV-5 isolated from the spontaneous cases of EPMF and have reproduced the signs of EMPF in the experimentally infected horses [75].
1.4.2 Asinine Herpesvirus Infections:

1.4.2.1 AHV-1 Infection:
AHV-1 is another member of the subfamily alphaherpesvirinae and tentatively under varicellovirus. The virus is also known as equine herpesvirus-6 (EHV-6). AHV-1 infection in donkeys causes very similar skin lesions caused by EHV-3 infection of horses on keratinized skin. Infected donkeys had erosive lesions on the muzzles and external genitalia as well as on the udders [76, 77]. In spite of disease similarities caused by EHV-3 and AHV-1, the restriction endonuclease and DNA hybridization analysis confirmed the distinct identity of AHV-1 [77].

1.4.2.2 AHV-2 Infection:
AHV-2 is a member of the subfamily gammaherpesvirinae and also tentatively known as equine herpesvirus-7 (EHV-7) [42]. The virus has been isolated from the leucocytes of a clinically normal donkey as well as also from nasal secretions of a mule [76, 78]. There is very little information available on this virus but it is believed that AHV-2 is distantly similar to EHV-2 and EHV-5 [76]. The characteristic disease symptoms caused by AHV-2 infections are not clear and the virus yet needs to be unequivocally correlated with disease [76].

1.4.2.3 AHV-3 Infection:
Browning et al. isolated one alphaherpesvirus from the nasal cavity of donkeys after high doses of corticosteroid treatment and the virus was named asinine herpesvirus-3 [76]. Although the sequence analysis study of glycoprotein G gene of AHV-3 showed considerable similarities (92% amino acid sequence similarity) with EHV-1, the restriction endonuclease fragment pattern confirmed the virus to be a distinct member of alphaherpesvirinae subfamily and is also named equid herpesvirus-8 (EHV-8) [76, 79, 80]. It is believed that donkeys are the primary hosts of this virus, but horses can also be naturally infected with AHV-3 [80]. AHV-3 infection primarily can cause afebrile rhinitis in donkeys but can also cause fever and rhinitis in horses [76, 80]. Like other alphaherpesviruses, AHV-3 can also be latent and can recrudesce at any time [80]. AHV-3
seronegative donkeys, when experimentally infected with the virus, showed afebrile rhinitis for 4 to 5 days, characterized by profuse serous nasal discharge which became mucopurulent later [76]. A complete genome sequence of a new strain of EHV-8, Wh by Liu et al revealed that the horse strain of EHV-8 has eight nucleotide and two amino acid substitutions in the gG gene when compared to other donkey strains of EHV-8 (AHV-3). Unlike EHV-1, AHV-3 has a restricted host range and under laboratory conditions AHV-3 does not grow well in equine fetal kidney (FEK) cells whereas EHV-1 grows rapidly producing high titers of infectious progeny virus [76]. Like EHV-1, AHV-3 also has 80 open reading frames which encode 76 unique proteins [80].

1.4.3 Gazelle Herpesvirus Infection:

While investigating the cause of death of eight Thomson’s gazelles from epizootic encephalitis in a zoological garden in Japan, Fukushi et al. isolated a herpesvirus which was serologically very similar to EHV-1 and EHV-4, and they named it Gazelle herpesvirus-1 (GHV-1) [81]. Although, based on the nucleotide sequences of the conserved region of gB and gG genes, the virus has about 95 per cent similarity with EHV-1 and EHV-8 and about 60 per cent similarity with EHV-4, DNA fingerprinting proved its separate identity and hence, GHV-1 was considered to be a new equine herpesvirus, equid herpesvirus-9 (EHV-9) [82]. In experimental conditions it has been shown that dogs, cats, goats, cattle, horses, mice, hamsters, pigs and non-human primate common marmosets (Callithrix jacchus) could successfully be infected with EHV-9 showing typical signs of fulminant encephalitis [82-84]. It is believed that equids (domestic as well as wild) are the natural primary hosts of EHV-9, and natural infections have been documented in equids such as Burchell’s zebras (Equus burchelli), Grevy’s zebras (Equus grevyi), Onagers, and other non-equid animals including cattle, deer, llamas, alpacas, gazelles, giraffes, and polar bears (Ursus maritimus) [81, 84-88]. Although the reservoir of this virus is not yet known completely, Burchell’s zebras and Grevy’s zebras have been shown to harbor the virus [82, 84]. Compared to EHV-1, EHV-9 has a wide host range and unlike EHV-1, EHV-9 does not cause encephalitis by vasculitis and hypoxic degeneration in adjacent neuronal tissues [82].

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Rather, cases of EHV-9 infections in wild equids and non-equids have shown that EHV-9 has tropism for respiratory as well as neural tissues and the infection primarily causes non-suppurative encephalitis characterized by neuronal degeneration with necrosis as well as intranuclear inclusion bodies in neuronal cells [81, 82, 84, 87]. However, the severity of EHV-9 infection in domestic horses is mild and only limited to transient fever, interstitial pneumonia and inapparent encephalitis [83].

1.5 Discovery of EHV-1

After the discovery of first virus, Tobacco Mosaic Virus in 1882, fifty years passed before William Wallace Dimock and Philip Edwards in 1932 first described that other than bacteria, different microorganisms could also cause epizootic abortion of mares [89]. In 1933 they first noticed that no bacteria could be isolated from the aborted fetuses. It was further demonstrated that bacteria-free tissue or body fluids from aborted fetuses could cause abortion in the pregnant mares [90]. From that study Dimock and Edwards at Kentucky Agricultural Experiment Station, University of Kentucky, Lexington, KY, concluded that a filterable agent was responsible for the epizootic abortion storms of mares in Kentucky [89, 90]. Three years later in 1936 Dimock and Edwards first documented few salient features that differentiated viral abortions from other abortions caused by Streptococcus, Salmonella abortus-equus and they named this abortions as ‘equine viral abortions’ [91]. In 1940 findings of inclusion bodies in liver cells and lung epithelial cells were considered to differentially diagnose ‘equine viral abortions’ from other abortions of infectious and non-infectious origins [92]. In 1941 Manninger and Csonitos in Hungary also documented the same symptoms of viral abortions found in Kentucky along with additional syndromes of respiratory diseases including mild fever [93]. Due to the respiratory symptoms they first considered that ‘equine viral abortions’ were caused by ‘influenza’ virus [93]. Later, in 1942 Salyi described both macroscopic as well as microscopic lesions in aborted fetuses in Hungary [94]. In the same year (1942), Dimock and Edwards also described the detailed clinical features of the viral abortion and the gross pathological changes of aborted fetuses [95]. Since
then, a number of animals including mice, guinea pigs, hamsters as well as different types of cells have been infected with EHV-1 under laboratory conditions to define the host range of this virus [96-100]. In an experimental study by Doll et al it was shown that equine abortion virus from the aborted fetuses can cause both respiratory disease and abortion in experimental mares [101]. Doll et al named the ‘equine abortion virus’ as ‘equine rhinopneumonitis virus’ to differentiate it from another virus, equine arteritis virus causing abortion [102, 103]. However, only in 1963 was the virus visualized under electron microscope and determined to be a member of herpesvirus group [34]. The rhinopneumonitis virus was then known as ‘equine herpesvirus type-1 (EHV-1)’.

1.6 Genome Organization Pattern of EHV-1:

After the complete genome sequencing of EHV-1 in 1992 by Telford et al information regarding the genome organization has been available [64]. Like other herpesviruses, EHV-1 has a linear dsDNA genome encapsidated in a thick-walled, spherical to pleomorphic, ~120 nm diameter, icosahedral capsid with 162 capsomeres [104]. The genome is about 150.224 kbp in length with an overall G+C content of 56.7 per cent and encodes 80 open reading frames (ORFs) [64, 105]. The viral DNA consists of a long unique region (UL) of 112,870 bp length which is flanked by two small inverted repeat sequences of 32 bp long, known as terminal repeats (TRL) and internal repeats (IRL) (Figure 1.3) [64]. The UL region is covalently linked to a short unique region (US) of 11,861 bp length which is also flanked by two large inverted repeat sequences of 12,714 bp lengths terminally and internally, known as terminal repeats (TRS) and internal repeats (IRs) respectively [64].
Figure 1.3: Genome Organization of EHV-1. The linear dsDNA genome of EHV-1 consists of a unique long (UL) and a unique short (US) region of 112.87 kbp and 11.861 kbp respectively. UL is flanked by a 32 bp long terminal repeats (TRL) and internal repeats (IRL) and US is also flanked by terminal repeats (TRS) and internal repeats (IRS) of 12.714 kbp each. ORFs 64, 65, 66, 67 are repeated inversely within the IRS and TRS and the two origin of replications (Ori s) are located in between ORF 64 and ORF 65. Another origin of replication (Ori L) is located within UL regions which encode ORFs 1 to 63. The US regions encode ORFs 68 to 76.

The US region of the genome can be oriented in two directions relative to the orientation of fixed UL region and this makes the presence of two equimolar mixtures of different isomeric forms of EHV-1 DNA [25].

The UL region encodes ORFs 1 to 63 and US region is predicted to encode ORFs 68 to 76 [64]. Four out of the eighty ORFs, ORF 64, ORF 65, ORF 66, and ORF 67 are duplicated inversely in IRS and TRS regions and hence, EHV-1 is predicted to have at least 76 unique genes [29, 64]. The ORFs are arranged compactly with little intervening sequences, without any extensive ORF overlap and few occurrences of exon splicing [25]. Like other alphaherpesviruses, the EHV-1 genome has 20 sets of short, tandemly reiterated DNA sequences, (8 in UL, 2 in US, 5 in each TRS and IRS) which are mostly present in the noncoding regions [29]. Beside the significant homology in the genome organization patterns with other alphaherpesviruses like HSV-1, VZV, pseudorabies virus (PrV) and BHV-1, EHV-1 is also assumed to have five unique genes encoded by ORF 1, ORF 2, ORF 67, ORF 71 and ORF 75 which have not been found in
any other herpesviruses to date [29]. Although the functions of these unique genes are unknown, ORF-1 encoded protein, membrane protein UL56 with the help of other unidentified viral protein(s) has been shown to down-regulate Major Histocompatibility Complex (MHC-I) molecules [106]. These unique genes are believed to be the responsible factor(s) that make EHV-1 distinct from other viruses of the same family [25].

1.7 EHV-1 Proteins:
EHV-1 has the capacity to encode 77 different types of proteins from 76 unique genes because of the splicing of ORF64 [64]. The architecture of a purified EHV-1 virion is very complex and comprises of at least thirty distinct polypeptides of which 6 polypeptides, encoded by genes 22, 25, 35, 42, 43 and 56, form the nucleocapsid core. The tegument of the EHV-1 consists of a number of proteins encoded by genes 8, 11, 12, 13, 14, 15, 19, 23, 24, 40, 45, 46, 48, 49, 51 and 76 (table 1.1, 1.2, and 1.3). Most of these tegument proteins also take part in virion morphogenesis, gene regulation and membrane fusion. The tegument proteins encoded by ORFs 19, 24 and 45 are also believed to degrade the host mRNA, help in capsid transport and in DNA encapsidation, respectively [105, 108]. At least seven proteins encoded by the ORFs 22, 25, 35, 35.5, 37, 42, 43 and 56 are involved in the formation of the architecture of the core and the nucleocapsid of the virion, of which the ORF 42 encoded major capsid protein is among the most abundant proteins found in the EHV-1 virion [25, 105].
Table 1.1: EHV-1 genes and their functions†

<table>
<thead>
<tr>
<th>EHV-1 GENE</th>
<th>START (Nt)</th>
<th>STOP (Nt)</th>
<th>HSV counterpart</th>
<th>Gene Product</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1298</td>
<td>1906</td>
<td>NA</td>
<td>Membrane protein UL56</td>
<td>Downregulate MHC-I expression, vesicular trafficking</td>
</tr>
<tr>
<td>2</td>
<td>2562</td>
<td>1945</td>
<td>NA</td>
<td>Membrane protein VI</td>
<td>Unknown</td>
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<tr>
<td>3</td>
<td>2841</td>
<td>3614</td>
<td>NA</td>
<td>?</td>
<td>Unknown</td>
</tr>
<tr>
<td>4</td>
<td>4249</td>
<td>3647</td>
<td>UL55</td>
<td>Nuclear protein UL55</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>5874</td>
<td>4462</td>
<td>UL54 (ICP27)</td>
<td>Multifunctional expression regulator</td>
<td>Gene regulation, inhibits premRNA splicing, post transcription regulation of genes, exports viral mRNA from nucleus</td>
</tr>
<tr>
<td>6</td>
<td>7042</td>
<td>6011</td>
<td>UL53</td>
<td>Glycoprotein K (gK)</td>
<td>Membrane fusion and virion morphogenesis</td>
</tr>
<tr>
<td>7</td>
<td>10301</td>
<td>7056</td>
<td>UL52</td>
<td>DNA helicase-primase</td>
<td>DNA replication</td>
</tr>
<tr>
<td>8</td>
<td>10300</td>
<td>11037</td>
<td>UL51</td>
<td>Tegument protein UL51</td>
<td>Virion morphogenesis</td>
</tr>
<tr>
<td>9</td>
<td>12115</td>
<td>11135</td>
<td>UL50</td>
<td>Deoxyuridine triphosphatase</td>
<td>Nucleotide metabolism</td>
</tr>
<tr>
<td>10</td>
<td>12084</td>
<td>12386</td>
<td>UL49A</td>
<td>Glycoprotein N (gN)</td>
<td>Membrane fusion and virion morphogenesis</td>
</tr>
<tr>
<td>11</td>
<td>12549</td>
<td>13463</td>
<td>UL49</td>
<td>Tegument protein VP22</td>
<td>Virion morphogenesis, possible RNA transport in uninfected cells</td>
</tr>
<tr>
<td>12</td>
<td>13595</td>
<td>14944</td>
<td>UL48</td>
<td>Transactivating tegument protein VP16</td>
<td>Virion morphogenesis, gene regulation by transactivating immediate early genes</td>
</tr>
<tr>
<td>13</td>
<td>15317</td>
<td>17932</td>
<td>UL47</td>
<td>Tegument protein VP13/14</td>
<td>Gene regulation by modulating VP16</td>
</tr>
<tr>
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<td>UL46</td>
<td>Tegument protein VP11/12</td>
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<td>Membrane fusion</td>
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<td>16</td>
<td>22851</td>
<td>21445</td>
<td>UL44</td>
<td>Glycoprotein C (gC)</td>
<td>Binds to heparan sulphate for cell attachment and also block neutralization by binding to complement factor c3b</td>
</tr>
<tr>
<td>17</td>
<td>24234</td>
<td>23029</td>
<td>UL43</td>
<td>Envelope protein UL43</td>
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<tr>
<td>18</td>
<td>25696</td>
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<td>DNA polymerase processivity subunit</td>
<td>DNA replication</td>
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<tr>
<td>19</td>
<td>26262</td>
<td>27755</td>
<td>UL41</td>
<td>Tegument host shut-off protein</td>
<td>Cellular mRNA degradation</td>
</tr>
<tr>
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<td>27894</td>
<td>UL40</td>
<td>Ribonucleotide reductase subunit 2</td>
<td>Nucleotide metabolism</td>
</tr>
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<td>31276</td>
<td>28904</td>
<td>UL39</td>
<td>Ribonucleotide reductase subunit 1</td>
<td>Nucleotide metabolism</td>
</tr>
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<td>32916</td>
<td>31519</td>
<td>UL38</td>
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<td>Capsid morphogenesis</td>
</tr>
<tr>
<td>23</td>
<td>33292</td>
<td>36354</td>
<td>UL37</td>
<td>36354 Tegument protein UL37</td>
<td>Virion morphogenesis</td>
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<tr>
<td>24</td>
<td>36588</td>
<td>46853</td>
<td>UL36</td>
<td>Tegument protein</td>
<td>Capsid transport</td>
</tr>
<tr>
<td>25</td>
<td>47311</td>
<td>46952</td>
<td>UL35</td>
<td>Capsid protein</td>
<td>Capsid morphogenesis and capsid transport</td>
</tr>
<tr>
<td>26</td>
<td>48230</td>
<td>47403</td>
<td>UL34</td>
<td>Nuclear egress membrane protein</td>
<td>Interacts with nuclear egress lamina protein</td>
</tr>
</tbody>
</table>
Table 1.2: EHV-1 genes and their functions† (continued)

| 27 | 48791 | 48369 | UL33 | DNA packaging protein UL33 | DNA encapsidation |
| 28 | 48763 | 50625 | UL32 | DNA packaging protein UL32 | DNA encapsidation and capsid transport |
| 29 | 50618 | 51598 | UL31 | Nuclear egress lamina protein | Interacts with nuclear egress membrane protein |
| 30 | 55184 | 51522 | UL30 | DNA polymerase catalytic subunit | DNA replication |
| 31 | 55453 | 59082 | UL29 | Single stranded DNA-binding protein | DNA replication, gene regulation |
| 32 | 59243 | 61570 | UL28 | DNA packaging terminase subunit 2 | DNA encapsidation |
| 33 | 61432 | 64374 | UL27 | Glycoprotein B (gB) | Membrane fusion, cell entry, cell-to-cell spread |
| 34 | 64578 | 65060 | NA | ProteinV | |
| 35 | 67093 | 65153 | UL26 | Capsid maturation protease | Capsid morphogenesis |
| 35.5 | 66142 | 65153 | UL26.5 | Capsid scaffold protein | Capsid morphogenesis |
| 36 | 68975 | 67212 | UL25 | DNA packaging protein UL25 | Possibly stabilizes the capsid and retains the genome inside capsid |
| 37 | 69897 | 69079 | UL24 | Nuclear protein UL24 | Possible neuropathogenicity factor |
| 38 | 69910 | 70968 | UL23 | Thymidine kinase | Nucleotide metabolism |
| 39 | 71192 | 73738 | UL22 | Glycoprotein H (gH) | Cell entry and cell-to-cell spread of virion |
| 40 | 76224 | 74632 | UL21 | Tegument protein UL21 | Virion morphogenesis, interacts with microtubule |
| 41 | 76793 | 77512 | UL20 | Envelope proteinUL20 | Membrane fusion and virion morphogenesis |
| 42 | 77703 | 81832 | UL19 | Major capsid protein; | Capsid morphogenesis |
| 43 | 82083 | 83027 | UL18 | Capsid triplex subunit 2 | Capsid morphogenesis |
| 44 | 84320 | 83148 | UL15 | DNA packaging terminase subunit 1 | DNA encapsidation |
| 45 | 84480 | 86600 | UL17 | DNA packaging tegument protein UL17 | DNA encapsidation |
| 46 | 86620 | 87732 | UL16 | Tegument protein UL16; | Possibly virion morphogenesis |
| 47 | 88917 | 87886 | NA | ? | |
| 48 | 88947 | 89900 | UL14 | Tegument protein UL14 | Virion morphogenesis |
| 49 | 89369 | 91153 | UL13 | Tegument serine/threonine protein kinase | Protein phosphorylation |
| 50 | 91135 | 92832 | UL12 | Deoxyribonuclease | DNA processing |
| 51 | 92784 | 93008 | UL11 | Myristylated tegument protein | Virion morphogenesis |
| 52 | 94472 | 93120 | UL10 | Glycoprotein M (gM) | Virion morphogenesis and membrane fusion |
| 53 | 94390 | 97053 | UL9 | DNA replication origin-binding protein | DNA replication |
| 54 | 97069 | 99324 | UL8 | DNA helicase-primase subunit | DNA replication |
Table 1.3: EHV-1 genes and their functions† (continued)

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†= Tables 1-3 are prepared on the basis of the data published in Allen et al [25], Ma et al [106], Telford et al [64], Davison et al [105] Kasem et Al [107]. $\ddagger$= ORFs are duplicated in the U5 regions of the genome; NA= Not available.

The ORFs 6, 10, 16, 33, 39, 52, 62, 70, 71, 72, 73 and 74 encode envelope glycoprotein K (gK), gN, gC, gB, gH, gM, gL, gG, gJ, gD, gI and gE respectively, homologues of which have been identified in other alphaherpesviruss like HSV-1 [109-115]. These glycoproteins are involved in different critical functions like attachment and entry of the virus into a cell, viral
determinants of cell tropism, cell-to-cell spread, pathogenesis and immune responses [25, 109, 112]. However, EHV-1 also has three unique glycoproteins, gp2, gp10, and gp21/22a [110]. The five glycoproteins, gB, gD, gH, gL and gK have been shown to be essential for viral replication while some other glycoproteins including gC, gE, gG, gI, gM and gp300 have been found non-essential for viral replication but involved in virulence of the virus [25, 116]. Neubauer et al using an EHV-1 mutant devoid of gB gene, have shown that gB is essential for the virus growth and cell-to-cell spread [117] in viro. It has been suggested that gB plays a major role in the entry of the virus by cell-to-cell fusion [118]. Monoclonal antibodies against gB protein have been shown to prevent the typical alphaherpesviral syncytium or cell-to-cell fusion in vitro [119]. A number of studies involving gB protein have documented its role as the most important immunogenic protein of EHV-1 [120, 121]. gC, another transmembrane glycoprotein, is the major determinant of virus attachment. gC of EHV-1 binds with the abundantly found glycosaminoglycans (GAG), and heparan sulfate (HS) present on the host cell surface and this binding helps concentrate the EHV-1 particles on the cell membrane which in turn potentiates the interaction with another glycoprotein, gD [122, 123]. This interaction of gC with gD is followed by another interaction with a specific cellular entry receptor that causes the stable attachment of the virus particle to the host cell membrane [124]. gC of EHV-1 is a very immunogenic protein that has been shown to induce high titers of neutralizing antibody and also cell mediated immune responses [125]. EHV-1 gC can also bind to the complement factor, C3b and inactivate it eventually allowing the virus to evade complement mediated lysis [126]. gD protein is absolutely necessary for the virus entry as well as virus-induced cell-to-cell fusion [124]. Other glycoproteins, gH and gL, form a heterodimer and gH is shown to be essential for direct fusion of the viral envelope with the host cell membrane, virus entry and virus-induced cell-to-cell fusion [127, 128]. On the other hand, the glycoprotein gE/gI complex is involved in the transmission of EHV-1 by cell-to-cell spread within the same host [116].

21
Like other herpesviruses, the gene expression pattern of EHV-1 is also temporally regulated resulting in the sequential expression of immediate early (IE or α) gene first, followed by early genes (E or β) and then the late genes (L or γ). Unlike HSV, EHV-1 has only one IE gene (ORF 64), the protein product of which is involved in the regulation of the expression of other genes of EHV-1 [25, 129].

The gene products of ORFs 7, 9, 18, 20, 21, 27, 28, 30, 31, 32, 36, 44, 45, 50, 53, 54, 57 and 61 are either origin-binding proteins, DNA polymerase or DNA packaging proteins which are involved in the replication and packaging of viral DNA [64, 105].

1.8 The Life Cycle of EHV-1:

EHV-1, during its life cycle, can undergo two different pathways- one for the lytic life cycle and the other for latency. During the lytic stage of the life cycle, EHV-1 actively replicates within the epithelial cells of the upper respiratory tract, endothelial cells of the blood vessels, and leucocytes and as a result, the animal harboring the virus sheds EHV-1 through the nasal route which can infect new horses [25]. New naive cells can be infected either by direct binding of virus or by cell-to-cell contact with infected cells [130]. The lytic replication cycle of EHV-1 is depicted in the figure 1.4. After the attachment and entry into the cells, EHV-1 uncoats its nucleocapsid which will be transported to nuclear pore complex. In the nucleus, the viral genes are sequentially transcribed and eventually translated into different viral proteins, some of which form new capsids. Once the genome replication is complete, new genomes are then encapsidated within the nucleus and then transported into cytoplasm where the progeny virus is enveloped from host cell membrane and buds out as progeny virion. The newly formed progeny virus can infect new uninfected cells where it can either choose lytic replication cycle or latency.
**Figure 1.4: Lytic Life Cycle of EHV-1.** EHV-1 enters into host cell by either direct membrane fusion or endocytosis. After entry virus uncoats its nucleic acid in the cytoplasm which is then transported to nucleus where viral gene expression starts. At the beginning IE gene (ORF64) will be expressed, then IE (α) protein will help to express E (β) genes. IE (α) and E (β) proteins both help in the expression of L (γ) genes. Viral genome replication starts within the nucleus immediately after the translation of E (β) genes. Next, some of the L (γ) proteins will form the capsid and the capsid assembly occurs within nucleus with the help of some chaperone proteins like pre-VP22a. After the assembly of capsids, the viral DNA concatamers are cleaved into unit length genomes by terminase enzyme with the additional help of pac1 and pac2 packaging signals. The assembled virions will acquire an outer membrane from nucleus which is lost during budding into cytoplasm where nucleocapsids acquire tegument proteins. Then it will get its secondary envelope from cytoplasm or endosomic vesicle and will be transported into cell surface and the nascent virus will be released by the fusion of vesicles with the plasma membrane.
1.8.1 The Lytic Life Cycle of EHV-1:

1.8.1.1 Virus Attachment and Entry:

EHV-1 can enter into the host cells by two major pathways: either through the direct fusion of the virus to the host cell membrane or through the cellular endocytosis or phagocytosis pathways depending upon the cell types [131-133]. The first step in the entry process of EHV-1 is the attachment to the host cell through receptor mediated binding. Multiple mechanisms and proteins of EHV-1 are involved in the attachment with host cell membranes since deletion of one particular protein cannot prevent virus attachment or replication completely [132, 134]. The initial EHV-1 particle attachment occurs through the interaction of gB and gC and host cell GAG proteoglycans. Glycoprotein C and also gB bind reversibly with heparan sulfate (HS), ubiquitously present on the mammalian cell surface [123, 134]. Osterrieder et al using a gC deletion mutant EHV-1 has shown that the virus can attach, enter and replicate both in non-equine cells (RK-13) and primary equine cells in the absence of gC although the titer of the virus is reduced to 5-10 fold for non-equine and 48-210 fold for primary equine cells [134]. A Study by Sugahara et al [123] has shown that mutation in gC gives the protein increased affinity towards HS and helps EHV-1 to adapt to different non-equine cells also. After the labile initial attachment, gD binds to specific cell-surface receptors which causes a conformational change allowing gD to interact with gH/gL and also gB, and this complex interaction makes the virus attachment stable and triggers the viral fusion with the host cell membrane [124]. gD of alphaherpesviruses has been shown to be indispensable for virus entry for HSV-1, PrV, and BHV but not for Marek’s disease virus or VZV which do not encode a gD homologue [135-139]. By studies involving CHO cells expressing EHV-1 gD or studies using monoclonal antibody against EHV-1 gD, or using a gD deletion mutant of EHV-1 it has been shown that gD is essential both for virus attachment and entry as well as for cell-to-cell fusion [124, 140, 141]. Unlike HSV-1, no specific receptor for gD of EHV-1 has been identified to date; although it is postulated that there is a novel entry receptor for EHV-1 gD other than HveA, HveB and HveC [142].
protein gH or gH/gL heterodimer also plays a major role in the entry and spread of EHV-1 from one infected to uninfected cells by cell-to-cell fusion and seems to be conserved among alphaherpesviruses [127].

Beside the classical pathway of entry by membrane fusion, EHV-1 also enters into certain cell types by endocytosis [132]. After the initial findings by Frampton Jr. et al studies by van de Walle et al have documented that EHV-1 can utilize cellular integrins such as αVβ5 with the ability to recognize the RSD motif [131, 132]. The interaction between the RSD motif present in gD of EHV-1 and cellular integrins like αVβ5 triggers the endocytosis of EHV-1 [131]. It has also been shown that EHV-1 enters into cells like PBMCs mainly by endocytic/phagocytic pathways while the virus enters into the endothelial cells by direct membrane fusion [131].

1.8.1.2 Transport of EHV-1 Capsid to the Nucleus:
Following membrane fusion the de-enveloped virion particles are deposited in the cytosol. In the case of endocytosis, the membrane of virion envelope fuses with the endosomal membrane inside the cell and eventually the naked capsids are released into the cytoplasm [143]. During this time some tegument proteins are dissociated from the capsid [14] and then the nucleocapsids are transported into the nucleus of the cell through the nuclear pore complex (NPC) [143]. This intracellular trafficking of viral nucleocapsids is achieved by the help of microtubules which are α and β dimers of tubulins with a structural orientation of positive end towards the periphery of the cell and the negative end towards the microtubule organizing center (MTOC) close to the nucleus [143-145]. EHV-1 nucleocapsid and some tegument proteins will be transported into the nucleus by the minus-end directed motor protein, dynein, along the microtubule. As has been shown by Frampton Jr. et al that in the presence of dynein inhibitor but not kinesin inhibitor, the EHV-1 replication is severely compromised [143]. EHV-1 triggers the acetylation of microtubules which causes its stabilization which is a primary requisite for dynein to transport the virion particles [143].
Whether by membrane fusion or endocytosis, in both cases, the cellular kinase Rho associated coiled-coil kinase 1 (ROCK-1) must be activated for the effective intracellular trafficking of nucleocapsid [132, 143] as inhibitors of ROCK-1 were shown to prevent the nuclear transport of capsid [143]. It is assumed that ROCK-1 will either mediate the interaction between virus and microtubules and/or other cytoskeletal components, or it can aid in the fusion of viral envelope with cellular membrane [143]. Once the nucleocapsids reach the NPC, a conformational change in NPC will allow the translocation of the viral genome as a densely packaged, rod shaped structure into the nucleoplasm through the dilated NPC [14, 130] and the empty capsids are then dissociated from NPCs and the genome transcription starts [146].

1.8.1.3 Expression of Viral Genes and Biosynthesis of Proteins:

Once the viral genome is in the nucleus of the host cell, the expression of EHV-1 genes, DNA replication, capsid assembly and DNA packaging will occur. As discussed earlier, expression of EHV-1 genes is temporally regulated and tightly coordinated. At the beginning, the IE (α) gene is expressed which triggers the expression of the E (β) genes, followed by L (γ) genes [129]. Six different regulatory proteins that control this cascade of gene expression are EHV-1 IE protein, EHV-1 infected cell protein 0 (EICP0), EICP22 or also known as IR4, EICP27, IR2 protein (splice variants of IE gene) and EHV-1 α-gene transinducing factor (ETIF or VP16) [147-152]. ETIF, the protein product of ORF12, is the only EHV-1 tegument associated late protein (γ2) that can transactivate the sole IE gene (ORF64) transcription without the help of any other proteins and this incident will trigger the EHV-1 gene transcription cascade. In HSV-1 the ETIF homologue α-TIF (VP16) with the help of a protein, host cell factor, interacts with the cellular transcription factor Oct-1 bound to the consensus sequence TAATGARAT (R= purine motif) present in the promoter regions of all the IE genes. A similar consensus sequence is also located within the EHV-1 IE promoter [147]. ETIF interacts with transcription factors TFIIB and dTAF40 and the interaction may form a preinitiation complex that starts the transcription of EHV-1 IE gene [147]. Structural analysis has shown that the IE protein has several domains
essential for the transactivation of viral genes such as acidic transcriptional activation domain, a serine rich tract that binds to the cellular EAP, nuclear localization signal and a DNA binding domain [152]. Once the IE (α) gene is expressed, it transactivates the promoters of the early genes EICP22 and EICP27 and enhances their transcription. EICP22 and EICP27 then synergistically transactivate the expression of IE promoter and the gene products of all these 3 proteins together synergistically transactivate the promoters of other early genes resulting in their enhanced transcription [150, 151]. The IE protein together with either EICP22 or EICP27 can transactivate the promoters of E (β) as well as L (γ) genes of EHV-1 [151]. The IE protein cannot transactivate the promoter of any of the late genes (γ1 or γ2) independently including the ETIF gene [147]. In contrast, EICP0 is a potent transactivator of all classes (α, β, and γ) of EHV-1 promoter and can strongly transactivate the promoter of ETIF gene too [149]. Surprisingly, the IE protein will repress the transactivation property of EICP0 genes [149, 153]. EICP0 and EICP27 synergistically can also transactivate the promoters of early and γ1 late genes while EICP0 and EICP22 transactivate γ2 gene promoters [149].

The IE (α) gene, however, trans-represses its own expression through the consensus IE protein binding site (5'-ATCGT-3') near to the TATA box and transcription initiation site [154]. On the other hand, the IR2 protein which is a truncated form of IE protein (amino acid 323 to 1487) binds with the promoter regions of IE, EICP0, EICP22, EICP27, thymidine kinase and late IR5 genes through the interaction with the transcription factor TATA box-binding protein (TBP) and represses the transcription of those viral genes [152].

1.8.1.4 Replication of Viral Nucleic Acid:

From the work done with other alphaherpesviruses like HSV-1, it is known that once the early proteins are synthesized, viral DNA replication will be started with the help of at least seven early proteins including the gene products of UL5, UL8, UL9, UL29, UL30, UL42 and UL52 [105, 155, 156]. In fact, the late genes are only produced in appreciable amount after a few
rounds of viral DNA replication [14]. After the viral DNA is deposited into the nucleus, it becomes circularized by DNA ligase IV/XRCC4 [104]. Simultaneously, some of the newly translated E (β) proteins which are essential for

Figure 1.5: Model for HSV-1 DNA Replication: In the nucleus, the linear genome is circularized by DNA ligase IV/XRCC4 and the circular DNA serves as template for the viral replication machinery. At the beginning theta-type replication is initiated at three redundant origins of replication oriS and oriL and later proceeds towards the rolling circle replication generating concatameric head-to-tail DNA. Lower figure shows the HSV-1 replication fork. The figure is adapted from Muylaert et al [156] with permission.
viral DNA replication, will bind to the template DNA to form the replication machinery and initiate the DNA replication which continues on a rolling circle mechanism (Figure 1.5) resulting in head-to-tail concatamers of viral DNA [157]. Although the mechanism of EHV-1 DNA replication is not well studied, the analogous process of HSV-1 DNA replication has been deciphered in much detail.

The viral DNA has 3 origins of replication, one in $U_L$ and two in $U_S$ regions (Figure 1.3). It is generally believed that at the beginning there will be bidirectional theta type of replication which makes circular daughter DNA that eventually undergoes rolling circle type of DNA replication, creating concatameric viral DNA [158]. The origin binding protein (product of UL9) will recognize, bind and activate the origins of replication, OriS and OriL, and unwind the viral DNA and this triggers other proteins to form a replisome consisting of DNA polymerase (protein product of UL30 and UL42), trimeric helicase-primase (protein product of UL5, UL8 and UL52) and ssDNA binding protein (protein product of UL29) [155, 159]. Besides the viral proteins, many host proteins and enzymes including DNA polymerase α-primase, DNA ligase, and topoisomerase II also play important roles in this process. The newly formed head-to-tail concatamers will later be cleaved to be packaged into individual capsids [159].

1.8.1.5 Assembly of Capsid and Encapsidation of Viral DNA:

The capsid assembly process in HSV-1, EHV-1 and even in cytomegalovirus is very similar [104] and takes place in the nucleus of the host cell [155]. The process starts only after the replication of viral DNA and synthesis of new capsid proteins and it is initiated by the oligomerization of the major capsid protein VP5.

Most of the proteins involved in the process are translocated into the nucleus themselves but VP5, VP23, VP26 require the additional help of other proteins such as VP19C or scaffolding protein pre-VP22a [155]. The capsid assembly continues as the oligomers of VP5 and pre-VP22a interact each other and to the edges of the growing capsid which is secured by VP23 [159]. Once
the viral DNA is packaged, the VP5-pre-VP22a complex is lost and then VP19C and VP23 proteins interact with each other to form partial capsids [155]. The triplex structures consisting of one molecule of VP19C and two molecules of VP23 link the individual capsomeres [155, 160]. Twelve copies of UL6 proteins forming the portal complex are added to the nascent capsid chain which becomes closed to form the procapsid [158, 160]. A series of structural transformations will lead to the maturation of procapsid into capsid [161]. After the assembly of capsids, the viral DNA concatamers are cleaved into unit length genomes by terminase enzyme with the additional help of pac1 and pac2 packaging signals [162, 163]. The viral protease cleaves and removes the scaffolding proteins VP21 and VP22a from inside the capsid and replaces them with newly replicated DNA genome [161]. Several other proteins such as UL6, UL15, UL17, UL28, UL32, UL33, UL36 and UL37 have also been implicated in the process of encapsidation of viral DNA [164-166].

1.8.1.6 Egress:
After the encapsidation of the viral DNA, the nucleocapsids then undergo the process of egress by the ‘envelopment-deenvelopment-reenvelopment pathways’ [167]. The newly formed nucleocapsids will acquire primary envelope from the inner leaflet of the nuclear membrane and bud out (Figure 1.6). The protein products of two conserved herpesvirus genes, UL31 and UL34, genes have been shown to be involved in primary envelopment [168]. Eventually the primary envelope will be lost (deenvelopment) and the nucleocapsids then move to the cytoplasm where they acquire the tegument [169]. Herpesviruses like HSV-1 have at least 15 different types of tegument proteins while VZV or CMV has been reported to have more than 15 tegument proteins [169]. Tegument proteins UL36 and UL37 play a major role in the maturation of the virion as the absence of those proteins abolishes the formation of the mature virion and its subsequent egress [169]. UL36 protein interacts with the major capsid protein, UL19 and forms the first layer of tegument around the icosahedral capsids [167, 169]. UL36 protein also interacts physically with UL37 protein which forms the second layer of tegument [169, 170]. The tegument protein UL48
(α-TIF) interacts with other proteins like UL41 and UL49 directly and also interacts with gB, gD, gH directly or indirectly [169]. The absence of UL48 severely impairs the tegumentation and affects virion morphogenesis downstream from envelopment and probably in the

**Figure 1.6: Summary of the Herpesvirus Egress Pathway.** In the nucleus, the newly synthesized herpesviral nucleocapsids will acquire primary envelopment by budding through the inner nuclear membrane and then capsids will undergo de-envelopment during its translocation towards cytoplasm where tegumentation occurs. Following to this, final envelopment occurs both in the cytoplasm or vesicles derived from trans-Golgi network (TGN) and at the endosome and eventually be transported to cell surface and the mature virions will bud out of the plasma membrane. The diagram is adapted from Mattenleiter [169] with permission.
virus assembly in cytoplasm [169, 171]. Von Einem et al have shown that ETIF, the HSV-1 UL48 homologue in EHV-1 has an indispensable role in the replication cycle of EHV-1 and besides its IE-gene transactivating property, it is also involved in secondary envelopment of the virion [171]. Although some tegument proteins are indispensable for the virus maturation, it has been noted that virion morphogenesis continues even in the absence of tegument proteins such as UL13, US3, UL41, UL46, UL47, and UL49 [169].

After the final tegumentation in the cytoplasm, the nucleocapsids will acquire the secondary envelope through protein-protein interactions occurring both in the cytoplasm or cytoplasmic vesicles derived from the trans-Golgi network (TGN) as well as at the endosomal network (Figure 1.6) [167, 172]. The nascent virions are transported to the cell surface by utilizing the cellular transport machinery and released by the fusion of vesicles with the plasma membrane (Fig 1.6) [172].

1.8.2 The Latent Life Cycle of EHV-1:

The initial lytic replication of EHV-1 in the respiratory epithelium of horses after the primary infection is followed by the onset of the latent stage of infection in which infected horses show no clinical signs of disease, virus shedding or cell associated viremia [25, 130]. Although the specific site of EHV-1 latency is not clear, it has been observed that the virus can remain latent in the sensory nerve-cell bodies of the trigeminal ganglia and also in lymphocytes, both in peripherally circulating lymphocytes as well as in lymphocytes found in draining lymph nodes [173-177]. The principal sub-population of the lymphocytes that can harbor latent EHV-1 are CD5+/CD8 T lymphocytes (>80%) though a smaller sub-population of CD5+/CD8+/CD4 T lymphocytes (20%) can also support latency of EHV-1 [178].

It is not clear what factors drive EHV-1 to enter into the latent cycle from the lytic one or what mechanisms actually control latency. It is, however, known that absence or interference in the activation of α-genes by VP16 protein can be related to the latency of HSV-1 [179]. Like
other alphaherpesviruses, the transcription of the EHV-1 genome during the latent infection is restricted, and only latency associated transcript (LAT) mRNA which is antisense to either the regulatory β-gene ORF 63 or the α-gene ORF 64 is transcribed. Because none of the viral proteins are expressed, the latently infected host cells escape immune surveillance and clearance [25, 130].

However, the latent horses become carriers for their lifetimes. These are potential reservoirs of infection in which the viral lytic cycle can be reactivated at any time when given favorable conditions like stress [16, 25]. It has been reported that in the field, stress induced by weaning, transportation, re-housing, infection by other viruses like EHV-2, or corticosteroid treatments have caused the reactivation of the EHV-1 from latency [25, 173, 176, 180]. Smith et al. have shown that treatment with interleukin-2 (IL-2) or gonadotropin causes the reactivation of latent EHV-1 from CD5^+/CD8^+ T lymphocytes through an indirect stimulation [178]. Viral factors also may play a major role in the reactivation process. A mutant EHV-1 devoid of thymidine kinase gene (TK^-) was shown to possess impaired reactivation capacity although the same mutant virus can achieve latency under experimental conditions [176]. During the reactivation process, the latent EHV-1 genome in a small subset of lymphocytes starts active transcription resulting in the expression of fusogenic viral glycoproteins on the cell surface, then cells undergo the transition from latency towards the active lytic replication cycle [181]. However, for the assembly and egress of infectious progeny virus following reactivation, lymphocytes carrying latent EHV-1 require fusion with permissive cells like endothelial cells [25]. Reactivation of latent EHV-1 from lymphocytes or trigeminal ganglia may cause infectious virus to reach the nasopharyngeal epithelium and result in viremia, abortion, or neurologic disease [25]. It is noteworthy that horses with reactivated virus may become silent virus shedders as reactivation often fails to cause apparent clinical signs of respiratory disease [25]. The cycle of latency and reactivation contribute importantly to the epidemiology of EHV-1 abortions since a majority of natural EHV-1 abortion have been documented even weeks or months after the cell-associated viremia ceases [25].
1.9 Epidemiology and Transmission:

EHV-1 infection is ubiquitous in nature among equids around the world and about 80-90% of horses have been estimated to get infected in the first two years of their life by either EHV-1 or its close relative EHV-4 [16]. Young horses and horses with compromised immune systems are more susceptible to EHV-1 infection [63]. Because of the capacity of EHV-1 to become latent, horses recover from the primary infection but become carriers for their lifetime and frequently shed infectious virus which helps to maintain the uninterrupted transmission cycle of EHV-1 to other susceptible animals [25, 63]. More than 80 million horses around the world have latent EHV-1 infection, which are the principal reservoir of infection and maintain the epidemiology of EHV-1 infection [25]. Studies reveal that about 54% of central Kentucky broodmares carry latent EHV-1 infection [182]. Although environmental contamination can cause indirect transmission, the environment may not be the principal reservoir since the virus is unable to survive even 35 days in the environment outside horse body [130].

EHV-1 is a highly contagious virus and it can be transmitted from one infected animal to others either by direct contact or indirectly [63]. Contact with virus-shedding horses with either acute infection or reactivated EHV-1 infection from latency, or virus-laden aborted fetuses or placenta are probably the major routes of EHV-1 transmission [63]. After contact of the horses with virus containing respiratory secretions, fomites or aerosols, the virus enters into the host initially through the upper respiratory tract epithelial cells [16, 25]. Naïve horses when exposed to EHV-1 infection shed virus from the respiratory mucosa for longer durations (as long as 15 days) compared to horses with experience of prior exposure or horses with reactivated EHV-1 from latency (2 to 3 days) [183, 184]. It is also documented that semen could be a source of EHV-1 infection and the virus may be transmitted via the semen during mating or through artificial insemination [185]. Most foals may get the infection for the first time during their first year of life, either before or after weaning [25]. EHV-1 infection has even been documented from foals
as young as 22 days old [186]. Unweaned foals probably get the infection from the reactivation of latent EHV-1 in their dams [25].

1.10 Pathogenesis and Clinical Signs:

1.10.1 Respiratory Disease:
The incubation period of the respiratory form of EHV-1 disease is very short, usually 1 to 3 days. It can be up to 10 days depending on the strain of virus, infecting dosage and immune condition of the host [25, 183, 187]. In the absence of mucosal antibody EHV-1 first infects and replicates in the nasal and nasopharyngeal epithelial cells although the conjunctival epithelium can also be infected after the direct contact with aerosol [25, 188]. In the case of intranasal experimental infection, virus can be detected in the infected epithelial cells of the nasopharynx, trachea and bronchi as early as 12 hours post infection [25, 189]. Subsequently, rounds of lytic replication of the virus cause the necrosis of the epithelial cells resulting in the formation of multiple erosions in the nasopharyngeal mucosa. These will cause the shedding of infectious virus in the first week of infection [25, 130]. Shortly the virus infection reaches to the underlying layer of lamina propria and infects the mononuclear leucocytes, which are drained into the draining lymph nodes where the virus is amplified by more rounds of lytic replication [189]. These infected leucocytes are transported to the circulatory system resulting in a cell-associated viremia that spreads the virus to the endothelial lining of the pregnant uterus as well as the central nervous system (CNS) causing abortion and neurologic disease, respectively [63, 190]. The identity of the sub-populations of the leucocytes that support the replication of EHV-1 during the cell-associated viremia is controversial [130]. Although primarily CD5+/CD8+ T lymphocytes are involved with viremia, EHV-1 DNA has been identified in CD4+ T lymphocytes, monocytes, and B cells during the acute phase of viremia [25, 130]. The cell-associated viremia, which is a prerequisite for abortion, develops as early as 3 days post infection and can linger up to 22 days [25, 63].
Figure 1.7: EHV-1 Pathogenesis. EHV-1 enters through the respiratory tract. It replicates in the respiratory epithelium and spreads to lamina propria where it infects lymphocytes and other immune cells. Infected cells are drained into lymph nodes where the virus is amplified by more rounds of replication. Infected cells then enter into the circulation causing viremia through which virus reaches to the blood vessels of uterus, brain and spinal cord where the virus replicates and causes inflammatory pathology resulting in abortion and EHM.

The magnitude of EHV-1 viremia ranges from 1 in $10^4$ to 1 in $10^7$ infected leucocytes in horse blood [63, 191]. However, in the case of the nasal and conjunctival routes of infection, the virus will disseminate to the trigeminal ganglion within 48 hours of primary infection [176]. Within 2-13 days post infection the virus can be detected in pulmonary leucocytes, epithelial and endothelial cells [189].

EHV-1 and EHV-4 infections principally cause disease of the upper respiratory tract which include rhinopharyngitis and tracheobronchitis, although infection with EHV-1 can also result in severe sequelae such as abortion, neonatal death, pulmonary vasculitis, ocular disease or
neurologic disease [25]. Often the respiratory form of disease may be asymptomatic especially in older horses with previous exposure to the virus or in the case of reactivation from latency [63]. In foals, EHV-1 infection involves bronchi and lungs making them vulnerable to secondary bacterial pneumonia [16]. In young horses, the clinical signs include depression, anorexia, fever, coughing, and initial bilateral nasal discharge. This is serous for first 2-3 days of infection and then becomes mucopurulent with desquamated respiratory cells along with inflammatory cells, as the condition progresses towards secondary bacterial infection [25, 63, 183]. In experimental infections with the AB4 strain of EHV-1, foals showed a biphasic fever (39-42 °C) with one peak at 1-2 days post infection and another at 6-7 days post infection [183]. Other clinical signs include neutropenia, lymphopenia, and progressive lymphadenopathy of submandibular lymph nodes as well as of retropharyngeal lymph nodes [192]. When the virus reaches the lower respiratory tract, the infected foals show the signs of bronchopneumonia [130]. The horse's immune system eliminates the virus from the respiratory tract within three weeks of the primary infection and one to two weeks of the subsequent infection or after reactivation from latency [183]. Even after clearing the infection from the respiratory tract, horses may develop some non-specific symptoms like bronchial hypersensitivity, chronic obstructive pulmonary disease syndrome or compromised athletic performance which is known as the ‘poor performance syndrome’ [16, 183, 193].

1.10.2 Abortion, Neonatal Diseases and Diseases of Stallions:
Abortion caused by EHV-1 is the most common type of infectious abortion in horses and it is estimated to be about 10% of all diagnosed abortions in thoroughbreds [194]. When viremia is established and the virus reaches to the gravid uterus, it infects the endothelial cells of the small arteriolar branches in the glandular layer of the endometrium at the base of microcotyledons, resulting in multifocal vasculitis [195]. The inflammation in the affected blood-vessels leads to thrombosis which further causes ischemic necrosis of the overlying microcotyledons and intercotyledonary stroma followed by the anoxic death of the fetuses leading to its expulsion.
The necrosis of the microcotyledons of the placenta sometimes causes the premature separation of the placenta which thereafter is expelled. In some cases, fetuses may be born alive even after the expulsion of placenta but shortly become affected with interstitial pneumonia. In most cases of EHV-1 induced abortions, virus can be isolated from the aborted fetuses, yet, in some experimental abortions induced by EHV-1, the virus could not be isolated from the fetuses. Virus is consistently isolated from the endothelial cells of the aborted fetuses, for which reason endothelial cells are thought to be the primary cells spreading EHV-1 to the adjacent parenchymal cells. The pathogenesis and the severity of the disease (abortion) depends on a number of factors like the level of viremia, the strain of virus, immune status of the animal and some host factors such as hormones. It has been shown by Mumford et al and others that virulent strains of EHV-1, such as AB4 or Army 183 strains cause higher abortion rates compared to less-virulent strains like V592. Even one neurovirulent strain, OH-03 or Findley strain of EHV-1 produced a lower abortion rate when compared with AB4 (another neurovirulent strain) in an intranasal experimental infection. On the other hand, host factors like prostaglandin hormone which is secreted at the uteroplacental interface are assumed to play roles to initiate the abortion.

EHV-1 infected mares may abort without any previous symptoms within 9 days to 4 months of infection although most abort within 21 days after infection. EHV-1 induced abortions could be sporadic as well as epidemic. The majority of the EHV-1 induced abortions occur in late gestation usually during the last 4 months. Normally the abortions do not compromise the mare’s future reproductive capability and the mares can become pregnant very soon after the abortion. Foals can be infected either within the uterus of the mare or can get the infections from their mothers just after they are born but both the cases lead to pneumonia, respiratory failure and death. Foals also suffer from gastrointestinal disease and neurological signs. Treatment is usually not effective against the foetal EHV-1 infections.
and most of the foals succumb to respiratory infection followed by secondary bacterial infection [208].

Although not well-studied, the reproductive ability of stallions could also be compromised by EHV-1 infections. Stallions have been reported to suffer from scrotal edema and loss of libido [209, 210]. Tearle et al have documented that stallions experimentally infected with EHV-1 showed a significant number of morphologically abnormal sperm cells and there was also evidence of shedding of infectious virus into the semen [211].

**1.10.3 Neurologic Disease:**
Although sporadic in nature the neurologic form of EHV-1 disease or equine herpesvirus myeloencephalopathy (EHM) is the most severe form of the infection. However, there has been an increase in the number of cases of EHM in recent years as has been documented in recent outbreaks in riding schools, racetracks as well as veterinary hospitals all over North America and Europe. This has caused the USDA-APHIS to list this disease as an emerging infectious disease [212, 213]. Unlike pathologies caused by other alphaherpesviruses like HSV-1, EHV-1 induced neurologic disease is developed as a result of the vasculitis of the blood vessels of the CNS. After the entry of the virus, and subsequent development of viremia, EHV-1 reaches to the different systems including the CNS where the virus establishes infection of endothelial cells of the arterioles of the brain and spinal cord. This causes an acute inflammatory response resulting in swelling, infiltration of inflammatory cells including lymphocytes releasing different types of cytokines such as tumor necrosis factor (TNF) with resulting tissue damage, deposition of immune complexes and formation of thrombi within the blood vessels. The vasculitis and thrombi lead to hypoxic degeneration and malacia of the surrounding nervous tissues of both the white and the grey matter with multifocal hemorrhages. The damage to the neural tissues also impairs blood flow and nutrient supply to the surrounding nervous tissues of the spinal cord and brain, aggravating the hypoxic degeneration leading to the death of neural cells. Although EHV-1 is an
endotheliotropic virus, it has also been occasionally isolated from the neural tissues of EHV-1 infected horses.

Outbreaks of EHM can occur as a squeal to respiratory disease or abortion or it can occur without the horse showing the signs of respiratory disease or abortion [214, 215]. There are probably multiple factors contributing to the development of EHM. Host factors like immune status of the animal, or vaccination frequency, virus factors like the strain of virus, and also environmental factors have been reported to influence the development of EHM. Although previously it appeared to be primarily a disease of pregnant and lactating mares, recent reports suggested that the disease can affect horses of any age or sex as has been evidenced by reports of outbreaks of EHM in foals, yearlings, geldings, mares and stallions [63]. Younger foals can develop the disease but older horses are more prone to develop EHM [216]. Allen (2008) reported that in an experimental study older horses (>20 years of age) with reduced frequency of cytotoxic T-lymphocyte (CTL) precursors were more susceptible to the development of EHM [217]. Some researchers also suggested that the season may also influence horses to develop EHM as most EHM outbreaks were reported to occur during late autumn, winter and spring [216]. A recent study by Nugent et al has linked a single point mutation from the nucleotide adenine (A) to guanine (G) in the DNA-polymerase gene of EHV-1 encoded by ORF30 at position 2254 (ORF30; A2254→G2254) to EHM [218]. This mutation changes the amino acid asparagine (N) to aspartic acid (D) at position 752 (N752→D752) of EHV-1 DNA-polymerase protein and this mutation enables the virus to cause high titer-viremia and develop EHM in horses [218]. It is interesting to note that EHV-1 with A2254 also has been reported to cause EHM in several cases while in other cases EHV-1 with G2254 has not produced any neurologic sign [219-221]. Moreover, a study by Kasem et al has suggested that the UL24 gene encoded by ORF37 is a determining factor for development of neurologic disease in a mouse encephalitis model [107].
These contradictory findings suggest that a single viral factor may not be responsible for development of EHM; rather, the disease is a multifactorial one.

The onset of neurologic signs of EHM starts within 6-10 days after the beginning of the respiratory tract infection; however, signs of EHM may appear as early as 24 hours following the onset of fever. Clinical signs may vary and are dependent on the location and severity of the lesions in the CNS [222]. The signs reach at peak within 48-72 hours after the onset of the symptoms of EHM [25]. Usually there is high fever (41.1 °C) before appearance of the signs of the neurologic disorder such as temporary ataxia, proprioceptive deficiency, stiffness and weakness of the limbs, swaying, stumbling and falling or complete paralysis [14, 25]. Although paralysis of the hind limbs is usually seen, quadriplegia has also been frequently reported. Normally the recumbency as a result of paralysis occurs within the first 24 hours of the onset of the symptoms of hind limb paralysis. Horses tilt their heads, and lose the sensation and sensory reflexes in the perineal area, inguinal areas and hind limbs although the appetite may remain normal [214]. Due to bladder dysfunction there may be urinary incontinence or retention of urine and signs of vulvar or penile flaccidity with cystitis are also reported. Horses will be severely depressed and the recovery time varies from several days to 18 months depending on the severity of the neurologic symptoms [215]. In the United States, the morbidity rate related to EHV-1 infection causing EHM ranges from 1% to 90% whereas the case-fatality rate ranges from 0.5% to as high as 40% [222].

Prognosis for the non-recumbent horses may be good but horses recumbent for more than 48 hours usually develop fatal complications like pneumonia, colic or bladder rupture, and eventually die in coma or convulsion [25, 222]. In some cases, however, severely recumbent horses also recover and the recurrence of the signs of neurologic disease from the recovered horses has not been reported [222].
1.10.4 Ocular Disease:
Hypervirulent strains of EHV-1 infection in foals may sometimes cause serious ocular disease, primarily chorioretinitis resulting in the visual impairment [223]. Within 3-5 weeks after respiratory tract infection by EHV-1, foals show the chorioretinal lesions which may be focal, multifocal, or diffuse [25]. Uveitis may also be seen in some foals after the outbreak of EHM [210]. Some reports also suggest that severe ocular infection can cause extensive retinal destruction leading to blindness [223]. Evidences suggest that diffuse retinal lesions can cause blindness while focal and multifocal lesions do not compromise the vision greatly [25].

1.11 Laboratory Diagnosis:
For the proper control of the disease, diagnosis at an early stage is of paramount importance. A physical examination of the infected horses for the clinical signs along with proper history is very important in the diagnosis of EHV-1 infection [16]. The respiratory form of the infection can be diagnosed at a laboratory by virus isolation on a number of EHV-1 sensitive cell lines such as rabbit kidney 13 (RK-13) cells, or baby hamster kidney cells (BHK) [214]. The level of EHV-1 specific serum antibody can be evaluated by enzyme linked immunosorbent assay (ELISA), or by the serum neutralization test. Development of an EHV-1 specific ELISA which can distinguish EHV-4 from EHV-1 has been very useful in the diagnosis of EHV-1 infection [224]. The most sensitive diagnostic tool for the diagnosis of EHV-1 is probably the real-time RT-PCR. Recently multiple real-time PCR assays have been developed not only to diagnose specifically EHV-1 but also to differentiate between neuropathogenic and non-neuropathogenic strains of EHV-1 on the basis of the presence of ‘G’ or ‘A’ nucleotide at 2254 position in DNA polymerase gene [225, 226]. Another important tool to diagnose EHV-1 is by immunofluorescence assay by using EHV-1 specific monoclonal antibodies [16]. After necropsy, immunohistochemical staining of paraffin embedded tissues is done to confirm the diagnosis of EHV-1 abortion as well as EHM cases [214]. Although the diagnosis of EHM is more difficult, a proper physical examination for the presence of neurological signs along with the history often helps tremendously. Clinical
specimens that are collected to diagnose EHM are nasal swabs, whole blood, cerebrospinal fluid, acute and convalescent serum [222]. Macroscopically, on Post-mortem examination brain and spinal cord may show a brownish patchy discoloration [214]. Microscopically, signs of vasculitis with ischemic and hemorrhagic infarction, perivascular edema and necrosis of parenchyma are seen [214].

However, differential diagnosis is often required from other similar infections like EHV-4 infection, equine influenza virus infection, or equine arteritis virus (EAV) infection for the respiratory form of disease; EAV infection for abortion; West Nile virus infection, Sarcocystis neurona infection for EHM in order to confirm EHV-1 infection.

1.12 Treatment and Supportive Care:
No specific treatment is available for EHV-1 induced diseases. However, supportive care such as good management practices to limit the spread of the infection, symptomatic treatment of infected horses, and use of antibiotics to control secondary bacterial infection can be very helpful to deal with EHV-1 infections. During EHV-1 induced rhinopneumonitis, phenylbutazone at 3 mg/kg body wt (bwt), PO, q 12h to 24h or, flunixin meglumine at 1.1 mg/kg bwt, IM, q 12h to 24h are the drugs of choice [16]. Trimethoprim-sulfadiazine preparation at 30 mg/kg bwt, PO, q 24 h for 7-10 days is used to prevent secondary bacterial infection [16]. In severe bacterial infections in young horses, broad-spectrum antimicrobials like amikacin, procaine Penicillin G, Cetiofur, or Ceftazidine can be used [16]. Horses with anorexia may need fluid electrolyte therapy. In case of paralytic EHV-1 infections, parenteral administration of corticosteroids (0.1 mg/kg bwt, IV, q, 24h) and nasogastric intubation of dimethyl sulfoxide (3 ml/kg bwt, q, 24h) may be used to reduce the inflammation [16]. In some cases of EHM or neonatal foal infection, nucleoside analogues such as acyclovir may also be used. Although use of acyclovir in vitro showed an inhibition in EHV-1 replication [227] but in vivo use of acyclovir in neurologic disease was not beneficial [228]. Beside acyclovir, valacyclovir also has been tried in
experimentally infected horses with EHV-1. Valacyclovir treatment, however, has not shown any significant reduction in clinical signs, virus shedding, and viremia of EHV-1 infected horses [229]. In case of EHM affected horses, cystitis is a common symptom which could be dealt with careful catheterization with antibiotics [14]. Laterally recumbent horses may be considered for euthanasia [16].

1.13 Prevention:
Complete elimination of EHV-1 infection from the herd is impractical because of the latent reservoirs of carrier horses that remain a continuous threat. However, the prevention of EHV-1 infection can be done by prophylactic immunization and good management practices in the herd.

1.13.1 Vaccination:
For all horses at risk of EHV-1 infection, vaccination is advised as a preventive measure. Maternally derived antibody through colostrum decreases with age and foals at the age of 5-6 months become maximally susceptible to infection [16]. Young foals at weaning should be provided two intramuscular injections of vaccines at an interval of 3 months, with the subsequent booster dose at every 3-6 months to prevent the respiratory form of disease [16]. Pregnant mares should be vaccinated to reduce the risk of EHV-1 induced abortions [16]. No vaccine is available to prevent either EHM or development of carrier state [230].

Both inactivated vaccines as well as modified live virus (MLV) vaccines are available and the immune response elicited against EHV-1 vaccination depends on the type of vaccines [230, 231]. The MLV vaccine induces both CTL mediated immunity and humoral immunity and have been found to reduce cell associated viremia [230]. On the other hand, inactivated vaccines have been shown to induce virus neutralizing antibody and also reduce viremia and nasal shedding of virus [231]. A DNA vaccine and also a recombinant vaccine using canarypox virus as vector expressing EHV-1 glycoprotein gB, gC, and gD were shown to suppress initial virus replication in the upper respiratory tract and the vaccines also induced neutralizing antibodies [232].
However, the vaccines provided a partial protection and did not protect EHV-1 challenged horses from cell-associated viremia [232]. In another study Soboll et al have observed that DNA vaccines comprising of EHV-1 glycoproteins gB, gC, and gD provided partial protection [233]. In spite of the promising results, none of the DNA vaccines targeting the glycoproteins provided horses with complete protection from EHV-1 abortions or EHM. Very interestingly, a recombinant vaccine consisting of IE gene of EHV-1 in modified live vaccinia virus vector provided significant protection from clinical disease as well as cell-associated viremia to EHV-1 challenged horses [234]. The vaccine induced both humoral and CTL mediated immunity but a compete protection from abortion and neurologic disease are yet to be achieved [234].

1.13.2 Management:
It has been reported that good managemental practices and sound vaccination strategy can reduce 75% of the EHV-1 induced abortions in the United States [235]. Management of herds should be primarily done to reduce the spread of epidemics [63]. Horses should be divided into small groups and segregated on the basis of distinct categories like weanlings, yearlings, new arrivals and transients [16, 63]. Any new horse to be included in the small groups should first be kept in quarantine or isolation for a period of 21 days [16]. Reduction of stress due to crowding, poor nutritional state, heavy parasite infestation, disruption of established social groups or inclement weather should be minimized [16]. Horses on prolonged transportation should also be kept for 21 days in isolation [16, 25]. Proper disinfection and hygienic practices must be followed to prevent the spread of EHV-1 infection during an already present outbreak [63].

1.14 Host Immune Responses to EHV-1:
After the discovery of EHV-1 in the early 1930s there have been numerous efforts over the decades by many scientists to characterize the protective immune responses against EHV-1 in order to find a suitable vaccine [230, 236]. EHV-1 infection will induce a short-lived protective immune response [237]. Both virus neutralizing (VN) as well as complement fixing (CF) antibodies have been found in the sera of EHV-1 infected horses as early as 2 weeks after
infection both in experimental as well as field conditions [27, 238]. VN antibody is EHV-1 type specific and gives protection for longer (maximum 1yr) than CF antibody (maximum 3 months) which cross reacts with EHV-4. Antibodies are directed principally against envelope glycoproteins, gB, gC, gD, gH and gp2 of the virus [110, 124, 239, 240]. gC and gD proteins of EHV-1 have been reported to have neutralizing epitopes [241, 242]. There is a controversy whether the humoral immunity of horses can protect them from clinical disease by EHV-1. It is however, true that the mucosal antibody, predominantly IgA, takes an important role in the protective response to EHV-1 infection by neutralizing the cytolytically replicating EHV-1 to reduce the nasopharyngeal virus shedding [243]. Re-infection with EHV-1 is very common in horses and, interestingly, even the presence of virus-specific high antibody titers cannot prevent horses from viremia during re-infection [243, 244]. Although VN antibody can reduce the signs of EHV-1 induced respiratory disease, it cannot protect the horses from abortion as well as neurologic disease, EHM [237].

As with other intracellular viruses, clearance of EHV-1 infection also depends on T lymphocytes, primarily on the CD8+ cytotoxic T lymphocytes (CTLs) which are the major effector arm of the cellular branch of the immune system [230, 233]. It has been demonstrated that there is a direct correlation between the frequency of CTL precursors (CTLp) and the level of protection in experimental EHV-1 infections [245]. Older ponies with high CTLp frequencies showed reduced signs of clinical disease after experimental EHV-1 infection [246]. Kydd et al. have reported that there is enhanced CTL activity and increased frequency of CTLp after experimental EHV-1 infection [245]. Since the identification of the importance of CTL-mediated immunity in the clearance of EHV-1, many attempts have been made to find the CTL target epitopes. Studies have shown that the immediate early (IE) protein encoded by ORF64 of EHV-1 serves as the CTL epitope which induces the virus-specific CTL-mediated killing of infected cells [233, 234, 247]. This CTL response to EHV-1 has been reported to be MHC class I serological
haplotype A3/B2 restricted [230, 233, 234, 237]. In another study, beside the IE protein, some
glycoproteins such as gC, gD, gI, and gL were also shown to have CTL epitopes, although the
findings were only seen in individual horses [248]. However, studies by Soboll et al did not find
any significant EHV-1 specific lymphoproliferative or CTL responses when they challenged
ponies with EHV-1 after DNA vaccination expressing gB, gC or gD [233].

1.15 Immune Evasion Strategies of EHV-1 and Other herpesviruses:
Despite these protective immune responses, complete clearance of this pathogen from the
infected host is yet to be accomplished because of many impressive immune evasion strategies
that EHV-1 possesses. The virus can interfere with all 3 principal immune effector mechanisms,
namely antibody dependent lysis, CTL-mediated killing and NK cell mediated killing of infected
cells [237].

1.15.1 Evasion from Antibody Mediated Immunity:
Herpesviruses are known to have co-evolved with their hosts for a long time. During this
period of evolution the virus evolved a number of immunomodulatory mechanisms to escape
from host immune surveillance to assure its existence throughout the host’s lifetime. Antibody
(Ab) dependent humoral immunity gives protection against viruses either by neutralization of the
cell-free viruses, opsonization or antibody dependent cell cytotoxicity (ADCC). After the
infection of the host cells by EHV-1, viral glycoproteins are synthesized and transported to the
surface of the cell membrane. In general, the virus specific antibody recognizes these
glycoproteins or viral antigens and triggers the host effector arms such as complement,
phagocytic cells and NK cells which then clear the virus from the host. After the recognition of
viral antigens, the complement component C1q binds to the Fc region of the virus-specific
antibody and initiates the subsequent cascade of reactions resulting in the destruction of the virus
infected cells. Similarly, NK cells as well as phagocytic cells like macrophages also express Fc
receptors that recognize the Fc domain of the virus-specific antibody bound to virus infected cells
and subsequently causes the lysis of the infected cells.
Although EHV-1 vaccination or infection induces a short-lived antibody responses, the recognition of EHV-1 infected cells is however, not optimal. EHV-1 can hide its antigenic proteins from being expressed on the surface of infected PBMCs as has been demonstrated by van der Meulen et al [249, 250]. More than 98% of EHV-1 infected PBMCs did not express any viral envelope proteins in their study even in the presence of cell-associated viremia. This prevents the virus infected cells from antibody mediated clearance and is a potent immune evasion mechanism adopted by EHV-1 [250]. Although late viral envelope glycoproteins were undetectable inside the infected PBMCs, the IE protein and early proteins such as ICP22 were detectable suggesting that viral replication in the PBMCs was restricted to the early phase [250]. It has, however, also been suggested that viral replication may not be restricted in PBMCs, but instead infected PBMCs expressing viral envelope proteins may be selectively removed from the circulation [250]. In both ways, interference in the expression of the envelope proteins on the surface of PBMCs makes the infected PBMCs resistant to the neutralizing effect of the circulating virus-specific antibodies. This helps the virus to escape from Ab-mediated killing of the infected cells and the viremic PBMCs can spread to different organs and systems. Interestingly, these infected PBMCs with restricted viral replication still have the capacity to transmit infectious viruses to susceptible cells like endothelial cells by direct contact [250]. It is possible that direct contact with susceptible cells may trigger yet unknown cell-signaling which initiates the onset of transition of the viral replication cycle from early to late phase [250].

On the other hand, a few PBMCs still express the viral envelope proteins but more than two-third of these cells cannot be cleared in the presence of complement and virus-specific Ab in vitro [249]. This escape from complement mediated lysis of infected cells could be attributed to the low efficiency of equine complement [251]. But this escape phenomenon may also be attributed to viral proteins which actively interfere with complement mediated cell lysis. Indeed, it has been shown that the envelope protein gC of EHV-1 binds to the C3 component of
complement interfering with downstream cascade events and this causes the virus to be resistant to complement mediated killing resulting in evasion of another major branch of the immune system [126].

1.15.2 Evasion from CTL mediated Immunity:

Because herpesviruses are intracellular parasites and are able to thwart Ab-mediated immunity, CD8+ cytotoxic T lymphocytes play a crucial role in clearing them from the host’s system. The CD8+ CTL-mediated immune system exerts its effect via the recognition of viral peptides presented by Major Histocompatibility Complex-I (MHC-I) molecules on the infected cell surface. In general, after the infection of the host cells by virus, viral proteins will be expressed and nascent proteins will be folded within the host cells. However, within the cytoplasm some of the nascent viral proteins, mainly misfolded proteins, will be cleaved by the host multi-subunit proteasome complex into smaller peptide fragments of 8-10 amino acids (aa) in length in an ubiquitin-dependent manner [252, 253]. These cleaved peptides are then transported into the endoplasmic reticulum (ER) via the Transporters associated with Antigen Processing -1 and -2 (TAP1 and TAP-2) [254]. Meanwhile, the newly synthesized MHC class I α chains will be transported into the ER for folding with the help of the chaperone protein, calnexin, and then will associate with β2-microglobulin (β2m) to form a heterodimeric complex (α:β2m) [253]. The short antigenic peptide fragments will then associate with the α:β2m heterodimer via TAP with the help of tapasin, another chaperone protein, to form a stable peptide-MHC-I complex which will finally be transported to the cell surface via the Golgi apparatus [254, 255]. It is also important to note that if MHC-I molecules do not associate with antigenic peptides they will be transported back to cytosol and be degraded by proteasome complexes in an ubiquitin dependent manner [256]. The peptide antigens (Ag) presented by MHC-I molecules on the infected cell surface will be recognized by the Ag-specific CD8+ T cells which will be subsequently activated and converted to effector cells known as CTLs [253, 256]. These CTLs
will then induce apoptosis in the infected cells, resulting in the clearance of the virus infected cells from the host’s system [253].

Even in the presence of these intricate host mechanisms, herpesviruses are still able to escape the host immune surveillance and co-exist with their hosts by adopting multiple immune evasion strategies. Using different continuous cell lines, such as equine embryonic lung cells, NBL-6 cells, or equine kidney primary fibroblasts, it has been shown that EHV-1 directly downregulates the surface expression of the MHC-I molecules on the infected cells in vitro [257, 258]. Rappocciolo et al reported that EHV-1 interferes with the cellular antigen presentation pathways by downregulating the cell surface expression of MHC-I molecules via the induction of enhanced endocytosis by early viral proteins [257]. Many herpesviruses inhibit the transport of peptides by interfering with the function of TAP, in order to interfere with the Ag presentation pathways. EHV-1 proteins were found to interfere with the peptide transport activity of TAP, causing the reduced availability of antigenic peptides in the ER for association with MHC-I molecules, which in turn results in reduced maturation and thereby downregulation of the cell surface expression of MHC-I molecules [237, 258]. Koppers-Lalic et al found that the EHV-1 protein pUL49.5 inhibits TAP and thus inhibits the expression of MHC-I molecules on the surface of infected cells [259]. Kurtz et al however, argued about two possible mechanisms for the downregulation of the surface expression of MHC-I molecules by early EHV-1 proteins [260]. Because EHV-1 utilizes MHC-I as an entry receptor, they suggested that it is possible that after EHV-1 binds to its receptor, that molecule will quickly be internalized along with the virus [260]. Another possible mechanism is that EHV-1 utilizes its receptor to enter into the cells and the MHC-I molecules are not internalized; rather, EHV-1 protein pUL49.5 suppresses further expression of the molecules on the cell surface [260]. Like EHV-1, other members of the genus Varicellovirus such as EHV-4, BHV-1 and PrV have been found to encode the novel TAP inhibitor, pUL49.5 protein, but the mechanism of inhibition is different for different viruses of the
same genus [261]. While pUL49.5 proteins of EHV-1 and EHV-4 interfere with the binding of ATP to TAP, the same protein of BHV-1 is found to trigger TAP for proteasomal degradation [261]. In a very recent study, it has been revealed that another novel EHV-1 phosphoprotein, pUL56 encoded by the ORF1, directly inhibits the surface expression of MHC-I molecules \textit{in vitro} and this inhibition was independent of pUL49.5 mediated inhibition of TAP [106]. It has been assumed that at the early stage of infection pUL56 will remove most of the MHC-I molecules from the surface of theinfected cells even before the loading of most viral peptides on to the binding grove of MHC-I molecules [106]. However, in the later stage of the infection, pUL56 will synergistically suppress MHC-I expression with pUL49.5 and interfere with the presentation of viral antigenic peptides to the CTLs [106]. The mechanism of this pUL56 mediated suppression of the surface expression of MHC-I molecules is yet to be determined but phosphorylation of pUL56 protein is not necessary for this action [106].

\textit{In vitro} EHV-1 infection was found to decrease the expression of MHC-I significantly on the surface of infected PBMCs [237, 250]. But \textit{in vivo}, infected and non-infected PBMCs from the same ponies experimentally infected with EHV-1 did not show a considerable difference in the expression of MHC-I molecules [237]. It is possible that this apparent difference was because of restricted EHV-1 replication \textit{in vivo} compared to \textit{in vitro} and also because only the absolute percentage of MHC-I positive PBMCs was considered, and the amount of MHC-I expression per infected PBMC was not considered in that particular study [237, 250].

Another strategy that herpesviruses could use to subvert the cell-mediated immunity is to destroy lymphocytes directly, and indeed experimental EHV-1 infection by Kydd \textit{et al} showed an acute and transient reduction in the number of T lymphocytes, both CD4+ and CD8+ T lymphocytes, but increased neutrophils in the lungs of infected horses at 2 days post infection (d.p.i.) [237, 262]. This reduction of the lymphocyte numbers could be either due to direct destruction of the lymphocytes, or selective migration of the immune cells causing overwhelming
infiltration of the neutrophils into the lung resulting in the decline in the number of lymphocytes [262]. Kydd et al. also reported an increase in the number of CD8+ T lymphocytes at 21 d.p.i. [262]. Another study by McCulloch et al. argued that the lymphopenia after EHV-1 infection may be either due to direct destruction of these lymphocytes by virus or partly may be due to the selective migration of different inflammatory cells in different times after infection [192]. In fact, there are multiple reports suggesting a decrease in the number of CD4+ as well as CD8+ T lymphocytes in the blood of infected horses [192, 263]. Beside these, EHV-1 may possess many other immune evasion strategies that are yet to be discovered to escape from CTL mediated eradication from the host’s system.

1.15.3 Evasion from NK cell-mediated immunity:

NK cells are a type of cytotoxic lymphocyte and are considered to be cells of the innate immune system. The cells have two types of receptors, activating and inhibitory receptors [264]. The activation of the NK cells depends on the intricate balance between these two types of receptors [264, 265]. The inhibitory receptors can sense the self MHC-I molecules on the surface of the antigen presenting cells and pass inhibitory signals to the cells [265]. But in general, during viral infections expression of MHC-I molecules on the infected cell surface is reduced or altered and that missing MHC-I molecules (known as ‘missing self’ hypothesis) triggers the activating receptors which in turn activate the NK cells to kill the infected cells [265]. The NK cells are considered to be evolved to supplement the function of CTLs which is inhibited by the reduced surface expression of different types of MHC-I molecules [265].

Many viruses including herpesviruses have been shown to develop specific strategies to evade NK cell-mediated immune surveillance [266]. Like other alphaherpesviruses, EHV-1 also has been shown to selectively downregulate the surface expression of certain types of MHC-I molecules while not affecting other types which indicates that the downregulation of MHC-I molecules is allele specific [257]. If that selective downregulation of MHC-I alleles does not
involve those molecules responsible for recognizing the inhibitory receptors, it is possible that the virus may easily evade NK cell-mediated detection and removal from the host’s system [257, 265]. Thus by selectively reducing the surface expression of MHC-I molecules on the infected cells, EHV-1 may escape both CTL as well as NK cell-mediated immunity.

1.15.4 Evasion from Cytokine Network:

Cytokines are small, hormone-like regulatory proteins (~25KDa) that are secreted from various cells in the body in response to different stimuli and act in autocrine, paracrine as well as endocrine manners via specific receptors present on various immune and non-immune cells. Cytokines play a critical role in control of the innate and the adaptive immune systems by controlling the development and expression of a broad array of immune responses [267]. Chemokines are pro-inflammatory cytokines that are chemoattractant in nature and are secreted in the early phase of the infection inducing direct recruitment of inflammatory cells and lymphocytes in adjacent cells [268]. Viruses have developed different strategies to evade the cytokines responses such as mimicking host cytokines or cytokine receptors, induction of inhibitory cytokines, or secretion of cytokine binding proteins [269]. Viral homologues of cytokines (virokines) or viral homologues of cytokine receptors (viroceptors) compete with their corresponding host cytokines or cytokine receptors respectively and modulate the host immune response by cytokines [270].

Experimental EHV-1 infections of ponies were shown to induce an inhibitory circulating cytokine, transforming growth factor β (TGF-β) that is reported to cause suppression of lymphocyte proliferation in vitro [271]. The subsets of immune cells that secrete active TGF-β after EHV-1 infection are alveolar macrophages, lymphocytes, and platelets [272]. Scientists also documented similar reports of EHV-1 mediated suppression of lymphocytes but unlike other studies, their reports did not find any circulating factor to be responsible for that immunosuppression [273-275]. EHV-1 also alters cytokine functions of the host with its envelope
protein, gG which is a viral homologue of chemokine binding receptors. The glycoprotein gG exists in two different forms, either in secreted soluble form or integrated into the cell membrane. The secreted gG binds to a broad range of chemokines and blocks the interaction of cellular cytokines with cellular receptors resulting in the suppression of chemokine mediated recruitment of inflammatory cells [276]. It has also been suggested that gG protein inhibits equine IL-8 mediated migration of neutrophils in the lungs of EHV-1 infected horses [277]. Furthermore, gG protein was also shown to suppress migration of murine macrophages by macrophage inflammatory protein 1α (MIP-1α) [131]. Thus, EHV-1 has been found to interfere with all major types of immunity of horses: cytokine-mediated immunity, Ab-mediated humoral immunity, CTL-mediated cellular immunity, and NK cell-mediated immunity.

1.16 Type-I Interferon and Host Immune Response:
Interferons are a group of biological regulatory proteins also known as cytokines which act as a first line of defense against many viral infections [278-280]. These cytokines are synthesized and secreted by the virus infected host cells and inhibit virus replication in the infected cells as well as nearby uninfected cells to induce a global antiviral state. While studying interference of influenza virus replication, Isaacs and Lindenmann first reported that a secreted antiviral factor induced by inactivated influenza virus could interfere with the replication of live influenza virus and they named the factor ‘interferon’ [281]. To date three classes of interferons (IFN), namely type-I, II and III, have been characterized on the basis of the amino acid sequences [279]. Type-I IFN includes IFN-α (multiple subtypes), β, κ, ε, τ, and ω; type-II includes IFN-γ and type-III includes λ1, λ2 and λ3 [278, 279]. Type-I and III IFNs are induced principally in viral infections by different types of cells including epithelial, fibroblast and dendritic cells. On the other hand, type-II IFN, which is also known as ‘immune IFN’, is expressed by the cells of the immune system, principally by activated T cells, NK cells and macrophages [282]. Type-III IFN functions by recruiting immune cells such as leucocytes, and macrophages to the site of inflammation and also contributes to induction of an antiviral state inside the host cell [282]. However, type-II IFN
is best known for its immune modulatory function rather than antiviral effect [278]. While type-I IFNs signal through IFN receptor 1 and 2 (IFNAR1 and IFNAR2), and type-II through IFN gamma receptor 1 and 2 (IFNGR1 and IFNGR2), type-III IFNs signal through IFN-λ receptor 1(IFN-λR1) and IL-10R2 [283].

**Figure 1.8: IFN-β Induction Pathways:** Viral PAMPs are recognized by different PRRs present either in the cytoplasm or endosome or on the outer cell surface. This recognition triggers the activation of different adapter proteins and kinases resulting in the activation of transcription factors IRF-3, and NF-κB, which then are translocated into the nucleus and associate with the promoter region of IFN-β gene. This causes an increased transcription of IFN-β gene which is then translated in the cytoplasm. IFN-β then signals through JAK-STAT signaling pathways resulting in the expression of IFN-α genes.

Type-I IFN induction by virus infected cells occurs in 3 phases- sensitization, induction and amplification [284]. In the sensitization or first phase, the viral motifs known as pathogen associated molecular patterns (PAMPs) are detected by the pattern recognition receptors (PRRs) present in the cells resulting in the signal transduction. These signals will converge to coordinate
activation of transcription factors like interferon regulatory factor 3 (IRF-3), and NF-κβ which induce IFN-β at a very low level [280, 285] (Figure 1.8). Two families of PRRs that play the major role in sensing the viral motifs are toll-like receptors (TLRs) and retinoic acid inducible gene-I (RIG-I) like receptors (RLRs). Among these PRRs, TLR-3, 7, 8 and 9, which are localized in endosomes and RLRs like melanoma differentiation-associated gene-5 (MDA-5), and Laboratory of Genetics and Physiology 2 (LGP2), which are localized into cytoplasm, are mostly engaged in viral PAMP detection. Most of the known PRRs signal through activation of IRF-3 by phosphorylation which forms dimers and translocates into nucleus, binds to the promoter of IFN-β and induces the transcription of IFN-β [284].

In the induction or second phase, the secreted IFN-β binds to its cognate receptors (IFNAR1 and IFNAR2) present ubiquitously on the cell surface (Figure 1.9). On binding of IFNs to their receptor subunits, there is heterodimerization and subsequent phosphorylation of tyrosine residues in the receptors [286]. This in turn triggers the activation of proteins in the receptor associated Janus kinase (JAK) family, such as JAK-1 and Tyk-2, by phosphorylation [286]. Activated JAKs phosphorylate signal transducer and activator of transcription-1 (STAT-1) and STAT-2 at tyrosine residues. These phosphorylated STATs associate with interferon regulatory factor 9 (IRF-9) forming a heterocomplex known as ISGF3 [287] (Figure 1.9). ISGF3 translocates into the nucleus where it binds to the interferon stimulated response elements (ISRE) of different IFN inducible genes including IRF-7 causing their enhanced transcription [287]. Activated IRF-3 forms a heterodimer with IRF-7, translocates into the nucleus and binds to the promoter of IFN-α genes causing their increased transcription [285].

In the amplification or third phase, the synergistic effect of IFN-α and IFN-β induces enhanced transcription of a diverse set of genes known as interferon stimulated genes (ISGs) which create a global antiviral environment within the host cell (Figure 1.9).
Type-I IFNs bind to their cognate receptors IFNAR1 and IFNAR2 causing the receptor dimerization which triggers activation of the JAK-1 and Tyk-2 protein kinases. The kinases then phosphorylate STAT-1 and STAT-2 molecules which then form a dimer and then translocate into the nucleus from the cytoplasm. Subsequently, the dimers associate with another protein, IRF-9 forming a trimeric complex, ISGF3, that binds to the interferon sensitive response elements (ISRE) to enhance the transcription of a number of ISGs.

**Figure 1.9: Type-I IFN signaling pathways.** Type-I IFNs bind to their cognate receptors IFNAR1 and IFNAR2 causing the receptor dimerization which triggers activation of the JAK-1 and Tyk-2 protein kinases. The kinases then phosphorylate STAT-1 and STAT-2 molecules which then form a dimer and then translocate into the nucleus from the cytoplasm. Subsequently, the dimers associate with another protein, IRF-9 forming a trimeric complex, ISGF3, that binds to the interferon sensitive response elements (ISRE) to enhance the transcription of a number of ISGs.

**1.17 Antiviral Immunity by Interferon Stimulated Genes:**

IFNs exert their antiviral effects through direct functioning of ISGs (Figure 1.9) which interfere with transcription and translation of viral genes, or promote apoptosis of the infected cells [284, 288]. Among more than 300 known ISGs, the most characterized type-I IFN induced genes are dsRNA dependent protein kinase R (PKR), 2’-5’-oligoadenylate synthetase (OAS), ISG-15, ISG54 and ISG-56 [280]. PKR activation requires binding to dsRNA, which is a byproduct derived during the replication of both DNA and RNA viruses. Activated PKR autophosphorylates, homodimerizes and then phosphorylates the eukaryotic translation initiation
factor, eIF-2α, inhibiting further translation of host cell proteins. In addition to this, PKR can induce NF-κB activation by phosphorylating IκB [289]. OAS activation also requires binding to dsRNA. This in turn converts ATP to 2’-5’-oligoadenylate which then activates a cytoplasmic enzyme, RNase L, leading to degradation of viral as well as cellular mRNAs and thus OAS inhibits virus replication [290].

ISG15 is, on the other hand, an antiviral protein that is upregulated dramatically in response to type-I IFN. ISG15 is an ubiquitin-like protein which covalently associates with many cellular proteins and ubiquitinylate those proteins for proteasome mediated degradation [291].

Another very important ISG of antiviral importance is ISG56 which happens to be the first IFN inducible gene to be discovered and cloned subsequently [292]. The ISG56 family of proteins includes ISG54 (p54), ISG56 (p56), ISG58 (p58) and ISG60 (p60) [293]. Human ISG54 and ISG56 have been reported to interact with translation initiation factor, eIF3 complex, and subsequently inhibit its ability to stabilize the ternary complex, eIF2-GTP-Met-tRNAi formed during the initial step of translation [293, 294]. Furthermore, recently it became known that ISG56 can also directly bind to viral proteins and inhibit replication of that virus. For example, ISG56 protein interacts with a key human papillomavirus (HPV) protein, E1 helicase and inactivates it [295].

Very recently one IFN inducible gene was identified while studying the genes induced by human cytomegalovirus (HCMV) infection and was named viperin (‘virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible’) [296, 297]. Viperin is a multidomain, multifunctional protein with 3 distinct domains: an N-terminal domain containing an amphipathic α helix, a central domain containing three cysteine residues organized in a CXXXXCXXC motif and a C-terminal conserved domain. Viperin is also known as radical S-adenosylmethionine domain containing 2 or RSAD2. The protein is induced in a variety of cell types by a number of
stimuli such as different DNA or RNA viruses, type-I, -II, -III IFNs, polyinosinic-poly cytidylic acid (poly I:C), or lipopolysaccharide (LPS) [297]. Viperin has been reported to interfere with the replication of a broad spectrum of DNA and RNA viruses such as HCMV, West Nile Virus, Dengue virus, and Influenza A virus through diverse mechanisms. It has been found that overexpression of viperin in fibroblasts interferes with the expression of HCMV structural proteins like gB, pp65, and pp28 that are required for viral maturation and assembly and thus inhibits replication of HCMV [296]. Viperin has also been found to bind and inactivate an enzyme, farnesyl diphosphate synthase required for the synthesis of cholesterol and other isoprenoid derived lipids [297]. As a result it can disrupt the lipid rafts present in the cell membrane and it can play a major role in the replication of viruses that use lipid rafts. It has indeed been found that overexpression of viperin inhibits the replication of influenza A virus by disrupting the membrane lipid rafts [298]. In addition to its antiviral property, viperin has also been suggested to be involved as a mediator in the intracellular signaling pathways that control immune responses. In plasmacytoid dendritic cells (pDC), viperin has been shown to mediate TLR7 and TLR9 mediated production of type-I IFN by recruiting signaling mediators IRAK1 and TRAF6 to lipid droplets [299].

Beside the ISG mediated innate immune response, type-I IFN also regulate adaptive immune responses [285]. IFN-α/β has been shown to upregulate monocyte derived dendritic cell (MoDC) maturation markers like MHC-I, MHC-II, CD80, CD86, CD40 and CD83 in vitro [300]. It has even been reported that monocytes cultured in GM-CSF + IFN-α/β rather than GM-CSF + IL-4 differentiate more quickly into DC suggesting a significant role of IFN-α/β in priming of T cells [300, 301].
1.18 Evasion of Type-I Interferon Response by Alphaherpesviruses:

As successful pathogens, viruses will always try to modulate the antiviral responses provided by type-I IFN. Multiple viruses have been reported to possess diverse mechanisms to evade the IFN-response. Some viruses interfere with global protein synthesis of the host cell; some viruses limit IFN induction by interfering with the production of viral PAMPs or their availability to PRRs; others inhibit IFN signaling pathways at multiple levels or interfere with the function of ISGs; or some viruses even develop replication strategies that are insensitive to IFN [279]. Herpesviruses are the master regulators of modulating host immune responses to their benefit. Because of their large coding capacity, herpesviruses have evolved multiple proteins to interfere with the type-I IFN response. Alphaherpesviruses like bovine herpesvirus-1 (BHV-1) and herpes simplex virus-1 (HSV-1) encode an IE protein in the infected cells known as ICP0 which has been shown to interfere at multiple steps of the type-I IFN response. ICP0 has a zinc RING finger domain at its amino-terminus which has E3-ubiquitin ligase activity [302, 303]. An intact zinc RING finger domain is necessary for the activity of ICP0 protein in most cases [285, 303]. Bovine ICP0 (bICP0) degrades IRF3 through its RING finger domain and it also interferes with IRF7 resulting in inhibition of IFN-β promoter activity as well as ISG promoters [302-304]. In contrary to BHV-1, HSV-1 ICP0 does not degrade IRF3 but sequesters IRF3 from binding to promoters of type-I IFN and ISGs [305, 306]. A variety of other mechanisms adopted by HSV-1 ICP0 have been reported by different researchers including inhibition of IRF7 mediated ISG induction as reported by Lin et al [307] and interference with STAT-1 activation as reported by Halford et al [308].

HSV-1 ICP27 is another multifunctional IE protein that has been shown to be involved at multiple steps in the viral lifecycle including viral gene expression and export of viral mRNAs [309-311]. The protein is also involved in repressing host transcription as well as mRNA stability leading to host shut off of protein synthesis [310]. Studies by Melchjorsen et al have revealed that ICP27 inhibits IRF3 activation leading to inhibition of IFN-β induction [306, 312]. ICP27 has
also been reported to inhibit STAT-1 activation and its nuclear translocation during wild type (WT) HSV-1 lytic infection, further establishing its role in interfering with type-I IFN response [313, 314]. HSV-1 infection to Vero cells causes secretion of a heat stable, IFN-antagonizing protein that inhibits nuclear translocation of STAT-1 in the infected as well as uninfected bystander cells [314]. Very interestingly mere expression of ICP27 protein of HSV-1 induces the cell to secrete the same antiviral protein with the ability to inhibit STAT1 activation as well as nuclear translocation [314].

A late protein of HSV-1, known as virion host shut off protein (Vhs), has been shown to be involved in inhibiting protein synthesis by degrading both host as well as viral mRNA[315]. Vhs is thought to interfere with the induction phase of the IFN response where IFN mRNA may be degraded by Vhs [316]. A Vhs-deleted HSV-1 has been reported to induce greater amounts of type-I IFN and ISGs in murine embryonic fibroblasts (MEF) compared to WT HSV-1 [317]. Vhs is also involved in the inhibition of the phosphorylation of STAT-1 [318, 319] and in the degradation of ISG transcripts [307, 320].

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 [321]. Peri et al found an increased induction of IFN-β mRNA and ISGs due to infection to monocytic cells with US3-deleted HSV-1, compared to decreased TLR3 mRNA and decreased dimerization of IRF3 in WT HSV-1 infection [322].

UL13 protein, which is also a serine threonine kinase of HSV-1, has also been reported to be involved in evading the type-I IFN response although the mechanism is not well characterized [323]. Besides, ICP34.5 and US11 have also been shown to be involved in interfering with the type-I IFN response [324, 325].
Some of the homologue proteins of HSV-1 and BHV-1 involving the type-I IFN-response have also been found in EHV-1 but their functions in the equine host in regard to the type-I IFN response are yet to be identified.

1.19 Statement of Problem and Hypothesis

EHV-1 continues to be a very important viral pathogen causing significant economic loss to the equine industry all over the world including the United States. As stated earlier, no existing vaccine can completely protect horses from abortion, EHM or reinfection. Moreover, within the past decade, the number of cases of EHM in EHV-1 outbreaks has been unusually high [212] which made this pathogen a great concern for the equine industry.

Most of the research characterizing the immune response to EHV-1 infection is focused on the adaptive immune system. Unfortunately, the interaction of EHV-1 with the innate immune system has not been well characterized. As that there is a strong correlation between neurologic symptoms and level of viremia [182, 226, 234, 326], the effective control of EHV-1-induced abortion and neurologic disease requires control of viremia [234]. Antibodies are inefficient in controlling the cell-associated viremia and even the CTL-mediated immune responses cannot clear the virus completely from the host [327]. To cause an abortion or EHM, infection of endothelial cells of the blood vessels of the uterus and nervous system by EHV-1 followed by lytic replication of EHV-1 in those cells is required. Characterization of the interaction of EHV-1 with the innate immune system may provide information about the mechanisms that EHV-1 employs in order to establish infection in endothelial cells, bypassing host innate immunity. This knowledge could be useful for designing of novel antiviral therapies against EHV-1 abortion or EHM. The innate immune responses to EHV-1 infection in endothelial cells are not well characterized and our current knowledge is rudimentary in this regard.

The most important mediator of the innate immune system that plays a major role in mediating potent antiviral responses to restrict viral replication irrespective of virus specificity is
IFN, especially type-I IFN. Type-I IFN are even capable of limiting some viral infections in the absence of adaptive immunity [328]. Although, most viruses like herpesviruses (alpha, beta and gamma), influenza viruses, and paramyxoviruses stimulate the induction of type-I IFN [285, 303, 305, 328, 329], viruses like porcine arterivirus and dengue inhibit its induction as a potential immune evasion mechanism [330, 331]. The type-I IFN response to EHV-1 infection has not been well-studied. Very few published reports are available [332] describing the type-I IFN response to EHV-1 infection. Edington et al reported the increased presence of type-I IFN [332] in the serum as well as increased production by cultured PBMC from the EHV-1 infected horses [332]. EHV-1 infection shows many distinct patterns of type-I IFN response compared to other herpesviruses. For example, pretreatment of fibroblasts with type-I IFNs cause reduction in the replication of alphaherpesviruses like HSV-1 in vitro [333]. Additionally, a combination of type-I and II IFN causes a greater reduction in HSV-1 replication in infected cells in vitro [329]. Again, in the cases of HSV infection in mice and humans and herpes zoster in man [332, 334, 335], low levels of type-I IFN correlate with higher spread of disease. On the other hand, Edington et al have observed that during EHV-1 infection in vivo, serum IFN levels are at their peak when viral pathogenicity is highest suggesting that high levels of serum IFN correlate with the severity of clinical disease [332]. Edington’s group also has demonstrated that EHV-1 is not susceptible to the action of type-I IFN [336]. Contrary to that report, Steinbach et al showed a marked reduction in the replication of EHV-1 due to the treatment of IFN-α [337]. This typical IFN response pattern raises some questions: does EHV-1 stimulate or inhibit type-I IFN production in vitro in cell culture and does this correlate with the in vivo findings of Edington et al [332]? If it stimulates the production of IFN, then how does the virus multiply in the presence of IFNs and why does the virus titer remains highest when serum level of IFN is highest? There are no detailed reports of the nature of the type-I IFN response against EHV-1 infection in vitro. Given the importance of the innate immune system in viral infection, it is important to characterize the EHV-1 interaction with the innate immune system, and especially the role of the
type-I IFN response in detail in equine endothelial cells (EECs). Finding the role of host innate factors in the pathogenesis of EHV-1 could also provide new and effective ways to control this pathogen.

The central premise of this work is that EHV-1 has mechanisms to evade the antiviral effects of type-I IFN response of the horse. It is apparent from the work of Edington et al that EHV-1 induces a type-I IFN induction in horses but the virus resists the antiviral effects of type-I IFN. A neuropathogenic strain of EHV-1, T953 will be used as a model virus for this study. The type-I response will be studied in EECs. Because, EECs will be considered to provide in vitro analogue of cellular responses responsible for EHV-1 abortion and EHM. As reviewed above that for the pathogenesis of EHV-1-induced abortion and EHM, replication of the virus in endothelial cells in horse is a key step [338]. To elucidate the mechanisms the sub-hypotheses of this study are

H1. EHV-1 infection will cause suppression of type-I IFN induction compared to uninfected cells, when treated with known type-I IFN inducer.

H2. EHV-1 will show reduced sensitivity to the antiviral effects of exogenous equine type-I IFN (rEqIFN-α) compared to VSV.

To test these hypotheses, the specific aims are

A1. To measure the induction of type-I IFNs in EECs infected with either T953 virus in the presence of known IFN-stimuli such as polyinosinic-polycytidylic acid (poly I:C) or absence of stimuli.

A2. To determine the effect of rEqIFN-α on the replication of EHV-1 in vitro in EECs compared to IFN-sensitive virus VSV.

The preliminary results from this study suggested an evasion of type-I IFN response by EHV-1 in vitro. Based upon those results, additional aims were also included to elucidate the mechanism of
suppression of type-I IFN response along with the viral genes responsible for that suppression in T953 infected EECs. The additional specific aims are

A3. To identify EHV-1 genes involved in the evasion of type-I IFN mediated immune response

A4. To identify the mechanism(s) used by EHV-1 to modulate the type-I IFN mediated immune response

The experimental approaches that will be taken to evaluate the specific aims are as follows: In specific aim A1, EECs will be infected with T953 or Sendai virus (SeV) at a multiplicity of infection (MOI) of 5 or co-infected with T953+SeV or T953+poly I:C and at different time points type-I IFN production will be evaluated by real-time RT-PCR as well as type-I IFN bioassay. The viral infection and replication will also be evaluated by indirect immunofluorescence assay (IFA) and plaque assay respectively. In the specific aim A2, equine cells will be pre-treated with rEqIFN-α to induce antiviral ISGs in those cells and then cells will be challenged with T953 virus at low MOI to see if the virus can evade the ISG mediated response. Virus replication in the IFN-treated cells will be the criterion to determine if the virus can evade type-I IFN response. In the specific aim A3, viral DNA polymerase will be inactivated using phosphonoacetic acid which in turn will block viral late gene expression and then the effect of late gene blocking on type-I IFN production will be evaluated in EECs. In the specific aim A4, EECs will be infected with T953 and different signaling pathways for IFN production such as IRF-3 and NF-κB signaling pathways will be studied to know if the virus is interfering with the activation process of those signaling pathways to suppress type-I IFN response. The signaling pathways by which type-I IFN functions (JAK-STAT signaling pathways) will also be investigated. In addition to this, role of T953 on the expression of different antiviral molecules induced by type-I interferon signaling will be elucidated in this study.

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CHAPTER II: MATERIALS AND METHODS

2.1 Antibodies, Cytokines and Other Reagents

All the antibodies that were used in this study for immunofluorescence assay (IFA) and Western blot (WB) have been listed Table 2.1.

Table 2.1: Antibodies used in this study

<table>
<thead>
<tr>
<th>Name of Ab</th>
<th>Source</th>
<th>Working Dilution/Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit IRF-3 (D6I4C XP)</td>
<td>Cell Signaling Technology, Danver, MA</td>
<td>1:200 (IFA), 1:1000 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit IRF-3</td>
<td>Santa Cruz Biotechnology, Dallas, TX</td>
<td>1:500 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit phospho-IRF-3</td>
<td>Cell Signaling Technology, Danver, MA</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit STAT-1 Ab</td>
<td>Santa Cruz Biotechnology, Dallas, TX</td>
<td>1:500 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit STAT-1 Ab</td>
<td>Origene, Rockville, MD</td>
<td>1:100 (IFA)</td>
</tr>
<tr>
<td>Anti-rabbit phospho-STAT-1</td>
<td>Cell Signaling Technology, Danver, MA</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit β-actin</td>
<td>Cell Signaling Technology, Danver, MA</td>
<td>1:1000 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit lamin A/C</td>
<td>GenScript USA Inc., Picataway, NJ</td>
<td>0.5 μg/ml</td>
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<tr>
<td>Anti-rabbit viperin</td>
<td>Abcam, Cambridge, MA</td>
<td>3 μg/ml</td>
</tr>
<tr>
<td>Anti-rabbit ISG56</td>
<td>Pierce through Thermo Fisher Scientific, Rockford, IL</td>
<td>1:2000 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit IκBα</td>
<td>Santa Cruz Biotechnology, Dallas, TX</td>
<td>1:500 (WB)</td>
</tr>
<tr>
<td>Anti-rabbit IE1</td>
<td>Dr. Dennis O’Callaghan [339]</td>
<td>1:1500 (IFA), 1:3000 (WB)</td>
</tr>
<tr>
<td>Anti-mouse gC</td>
<td>Dr. George P. Allen [110, 241]</td>
<td>1:100 (IFA), 1:1000 (WB)</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>Cell Signaling Technology, Danver, MA</td>
<td>1:3000 (WB)</td>
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<tr>
<td>conjugated to HRP</td>
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<tr>
<td>Goat anti-mouse IgG</td>
<td>Cell Signaling Technology, Danver, MA</td>
<td>1:3000 (WB)</td>
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<tr>
<td>conjugated to HRP</td>
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<td></td>
</tr>
<tr>
<td>Goat anti-rabbit IgG (H+L)</td>
<td>Santa Cruz Biotechnology, Dallas, TX</td>
<td>1:300 (IFA)</td>
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<tr>
<td>conjugated to FITC</td>
<td></td>
<td></td>
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<tr>
<td>Texas Red goat anti-mouse IgG (H+L)</td>
<td>Life Technologies, Grand Island, NY,</td>
<td>1:400 (IFA)</td>
</tr>
</tbody>
</table>

For the detection of EHV-1 infection, anti-rabbit IE1 mAb (1:1500 for IFA, 1:3000 for WB) was received as a kind gift from Dr. Dennis O’Callaghan, Louisiana State University. Anti-mouse
For the detection of EHV-1 infection, anti-rabbit IE1 mAb (1:1500 for IFA, 1:3000 for WB) was received as a kind gift from Dr. Dennis O’Callaghan, Louisiana State University. Anti-mouse EHV-1 gC mAb was developed by the late Dr. George P. Allen at Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY 40503 and was kindly provided by Dr. Udeni B.R. Balasuriya, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY 40503. Polynosinic-polycytidylic acid (poly I:C), was purchased from InvivoGen (CA, USA) and phosphonoacetic acid (PAA) was purchased from Thermo Fisher Scientific, Rockford, IL, USA. Recombinant equine IFN-α (rEqIFN-α) was purchased from Kingfisher Biotech, Saint Paul, MN, USA.

2.2 Culture of Cells

All the cell culture medium and reagents used in this study are enlisted in the table 2.2.

Table 2.2: List of cell culture reagents used in this study

<table>
<thead>
<tr>
<th>Cell culture reagents</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>Eagle’s Minimum Essential Medium (EMEM)</td>
<td>Mediatech Inc., Manassas, VA</td>
</tr>
<tr>
<td>Minimum Essential Media (MEM)</td>
<td>Invitrogen, Carlsbad, CA</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM)</td>
<td>Mediatech Inc., Manassas, VA</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate-Buffered Salaine (D-PBS), pH 7.4</td>
<td>Mediatech Inc., Manassas, VA</td>
</tr>
<tr>
<td>Fetal Calf Serum (FCS)</td>
<td>Hyclone Laboratories Inc., Logan, UT</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>Hyclone Laboratories Inc., Logan UT</td>
</tr>
<tr>
<td>Penicillin-Streptomycin</td>
<td>Gibco, Carlsbad, CA</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>Gibco, Carlsbad, CA</td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>Gibco, Carlsbad, CA</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Gibco, Carlsbad, CA</td>
</tr>
<tr>
<td>Non-essential Amino Acids</td>
<td>Gibco, Carlsbad, CA</td>
</tr>
<tr>
<td>Trypsin-EDTA</td>
<td>Mediatech Inc., Manassas, VA</td>
</tr>
<tr>
<td>Rabbit Kidney-13 (RK-13) Cells</td>
<td>ATCC, CCL37, Manassas, VA</td>
</tr>
<tr>
<td>Madin-Darby Bovine Kidney (MDBK) Cells</td>
<td>ATCC, CCL22, Manassas, VA</td>
</tr>
<tr>
<td>African Green Monkey Kidney Cells (Vero Cells)</td>
<td>ATCC, CCL81, Manassas, VA</td>
</tr>
</tbody>
</table>

Equine endothelial cells (EECs) from pulmonary artery [340] were kindly provided by Dr. Udeni B.R. Balasuriya at Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY, USA. EECs were maintained as confluent monolayers in 150-cm² cell culture flasks in Eagle’s Minimal Essential Medium (EMEM) with 10% fetal calf serum, 100 U/ml
penicillin-streptomycin, 1 mM sodium pyruvate, 200 mM L-glutamine and sodium bicarbonate (0.6 g/L) at 37°C in the absence of CO₂. When confluent, the cell monolayer was washed 2x with 30 ml D-PBS and then trypsinized with 2 ml Trypsin-EDTA at 37 °C for 2-3 min. Cells from one 150-cm² cell culture flasks were then resuspended in 100 ml enriched DMEM containing 10% fetal calf serum, 100 U/ml penicillin-streptomycin, 0.1 mM non-essential amino acid and 200 mM L-glutamine and were propagated into four 6-well plates. When needed (such as for immunofluorescence study) EECs were also grown onto cover slips in 24-well cell culture plates in DMEM containing 10% fetal calf serum, 100 U/ml penicillin-streptomycin, 0.1 mM non-essential amino acid, and 200 mM L-glutamine.

Rabbit kidney-13 cells (RK-13 cells), African Green Monkey Kidney cells (Vero cells) and Madin-Darby bovine kidney cells (MDBK cells) were maintained in 75-cm² cell culture flasks in EMEM supplemented with 10% fetal bovine serum, 100 U/ml Penicillin-streptomycin, 1 mM sodium pyruvate and 200 mM L-glutamine.

2.3 Virus Strains
The equine herpesvirus used in this study was the neurovirulent T953 strain of EHV-1 (also known as Findlay strain or OH’03 strain of EHV-1). The virus was originally isolated from the nasopharyngeal swab of a horse suffering from EHV-1 induced quadriplegia during the extensive neurologic disease outbreaks that occurred at the Veterinary Medical Teaching Hospital, at The University of Findlay, Ohio, USA in January, 2003. [213]. The virus was isolated and archived by the late Dr. George P. Allen and it was kindly provided by Dr. Udeni B.R. Balasuriya at the Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY. The neurovirulence phenotype of EHV-1 T953 strain was further confirmed by experimental inoculation of horses with low passaged EHV-1 T953 strain by the late Dr. George P. Allen [217]. The virus was originally isolated and propagated on the equine fibroblast cell line
at a low MOI. For this study, the virus was amplified in EECs and passage 3 (P3) of this virus was used.

Another neuropathogenic strain of EHV-1, Ab4 was isolated from a mare suffering from paresis in England in 1980 [341]. The recombinant Ab4 mutant virus which was generated by deleting ORF1 and ORF2 genes (Ab4ΔORF1/2) and WT Ab4 [342] were kindly provided by Dr. Nikolaus Osterrieder, Institut für Virologie, Freie Universität Berlin, Berlin, Germany. Sendai virus (ATCC VR907, Cantell Strain) was purchased from Charles River Laboratories International Inc., Wilmington, MA, USA. The recombinant vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP) was kindly provided by Dr. Adolfo García-Sastre (Mount Sinai School of Medicine, NY, USA).

2.4 Preparation of Viruses

To prepare a stock of EHV-1 virus, EECs in 175 cm² cell culture flasks were infected with EHV-1 T953 at an MOI of 0.1. When nearly 100% cytopathic effect (CPE) was shown, viruses were harvested by three cycles of freeze-thaw of the infected flasks. The viruses were clarified at 2000 g for 30 min at 4°C, filtered through 0.45 μm cellulose acetate membrane filter (Thermo Fisher Scientific, Pittsburgh, PA) and the tissue culture fluids (TCFs) containing the virus was aliquoted and preserved at -80°C as stock virus. From this stock, the virus was again amplified in EECs and clarified as stated and TCFs were further purified by ultracentrifugation at 100,000 x g for 4 h at 4°C through a 20% sucrose (in D-PBS) cushion using L7 Ultracentrifuge machine (Beckman Coulter, CA, USA). To maximize the titer of the virus, each pellet was then resuspended in minimum volume (100-200 μl) of sterile DMEM with 2% FBS, sonicated briefly (30 sec x 3 cycles, with 1 min rest in between a cycle) and aliquoted at 100 μl each in 1.5 ml eppendorf tubes to save at -80°C. The viruses were titrated on RK-13 cells by plaque assay. Ab4 wild type (WT) and Ab4ΔORF1/2 viruses were propagated and amplified on EECs and purified in the same manner as T953 was done.
UV inactivation of EHV-1 T953 strain was achieved by using UV Stratalinker 1800 (Stratagene). Briefly, 1 ml of TCF containing the T953 virus was placed in a 35 mm petridish and was exposed to the ultra-violet rays with a wavelength of 254 nm for 30 min on ice from a distance of 10 cm from the UV bulb. After the UV treatment, the virus was propagated on a monolayer of RK-13 cells to confirm the inactivation.

Sendai virus was inoculated into the allantoic cavities of ten day old specific pathogen free embryonated hen eggs, which were incubated at 37°C for 72 h. Allantoic fluids were harvested and clarified at 2000 x g for 30 min at 4°C and then purified by ultracentrifugation at 100,000 x g for 4 h at 4 °C through a 20% sucrose (in D-PBS) cushion using L7 Ultracentrifuge machine (Beckman Coulter, CA, USA). The pellet was resuspended in calcium-magnesium free D-PBS, and aliquoted at 100 μl each in 1.5 ml eppendorf tubes and saved at -80 °C for future use. The virus was titrated by plaque assay on Vero cells.

VSV-GFP was amplified in MDBK cells by infecting confluent layers of MDBK cells in 800 cm² Corning roller bottles (Sigma Aldrich, St. Louis, MO, USA) at an MOI of 0.5. When approximately 100% CPE was shown, viruses were harvested by three cycles of freeze-thaw of the infected roller bottles. The viruses were then clarified at 2000 g for 30 min at 4 °C, filtered through a 0.45 μm cellulose acetate membrane filter, and TCFs containing the virus were aliquoted and saved at -80 °C as stock virus. This stock virus was again amplified in MDBK cells and clarified as stated and TCFs were further purified by ultracentrifugation at 100,000 x g for 4 h at 4 °C through a 20% sucrose (in D-PBS) cushion using L7 Ultracentrifuge machine (Beckman Coulter, CA, USA). The pellets were then resuspended in minimum volume (100-200 μl) of sterile MEM, sonicated briefly (30 sec x 3 cycles with 1 min rest between each cycle) and aliquoted at 100 μl each in 1.5 ml eppendorf tubes to save at -80 °C. The virus was titrated on MDBK cells by plaque assay.
2.5 Virus Infection of EECs

EECs were cultured in 175 cm² tissue culture flasks and were propagated in either 6-well or 24-well plates before infection. At confluency, EECs were washed twice with sterile D-PBS and then infected with either T953, SeV or combination of both of the virus at an MOI of 5. During infection in a 6-well plate, virus was added in a total volume of 200 μl and in case of 24-well plate virus was added in a total volume of 100 μl. The virus was adsorbed onto the EECs for 1 h at 37 °C, 5% CO₂ with an intermittent gentle mixing of the plates at every 10 min interval. Cells were mock infected with virus diluent (DMEM+2% FBS). After the incubation, the plates were washed again with D-PBS and the growth medium was added. The infected plates were then incubated at 37 °C with 5% CO₂ in a humidified chamber for the indicated time points and different assays were performed as will be described below.

2.6 Plaque Assay

The virus was titrated by plaque assay as described by McCollum et al with little modification [343]. Briefly, RK-13 cells or MDBK cells or Vero cells were propagated in 6-well plates. Then the confluent monolayers were washed twice with D-PBS and infected with 10 fold serial dilutions (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, and 10⁻¹⁰) of cell culture supernatants containing virus. 10 fold serial dilutions of virus were made by adding 100 μl of virus to 900 μl of sterile MEM. For each dilution of the virus, duplicate wells were infected. The virus was let to adsorb for 1 h with intermittent shaking at 10 min interval at 37°C inside an incubator in presence of 5% CO₂. Next, the plates were washed twice with PBS (pH 7.4) to remove the unadsorbed viruses and the growth medium (enriched EMEM with 10% FBS) with 0.75% carboxy-methyl cellulose (CMC) (Sigma Aldrich, St. Louis, MO) were added and the plates were further incubated for 96 h at 37°C, 5% CO₂. Then the growth medium was removed and the plates were stained with 1% crystal violet solution in buffered formalin (10%) for visualization of plaques.
The titers were calculated by counting the number of plaques and multiplying by the dilution factors. Finally, the titers of the viruses were expressed in plaque forming units/ml (PFU/ml).

2.7 Infectious Center Assay

The infectious center assay (ICA) is normally used to calculate the proportion of cells within a culture of sensitive cells which are infected with virus. This assay was performed in this study to know if rEqIFN-α treatment of EECs makes the cells resistant to virus entry following infection with T953 virus. During the early phase of the infection or eclipse phase, there is no free virus and as a result no CPE is found within the indicator cells.

EECs were propagated into 6-well plates and at about 90% confluency, the cells were treated with rEqIFN-α at 1000 IU/ml for 24. One group of EECs was also left untreated. After IFN-treatment the cells were washed 2X with D-PBS and challenged with T953 at an MOI of 0.5 and incubated at 37 °C for 1 h in presence of 5% CO2. After virus adsorption, the cells were washed 2X with D-PBS, trypsinized and resuspended as single cell suspensions in growth media. Cells were then counted and equal numbers of cells (2x105 cells in total volume of 200 ul) were plated in duplicate wells for each dilution onto confluent monolayers of RK-13 cells (indicator cells). The cells were then overlaid with 0.75% CMC in EMEM complete growth media and incubated at 37 °C with 5% CO2 for 96 h. After that the growth medium was removed and the plates were stained with 1% crystal violet in buffered formalin for 2 h at RT. The plates were washed under running tap water and the plaque numbers were counted. The number of plaques which were formed on the indicator cells indicated the number of EECs that were originally infected with T953. Then the infected cell numbers per 2 million EECs that were originally infected with the virus were determined.
2.8 RNA Extraction and Real-Time RT-PCR Assay

Total cellular RNA was extracted from mock or T953 infected or otherwise coinfected (T953+SeV or T953+poly I:C or T953+EqIFN-α) EEC samples from 3 h, 6 h, 12 h and 18 h post infection (p.i.) using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. The possible genomic DNA contamination was removed by in-column treatment of RNA with DNase-I (RNase free DNase set, Qiagen, Valencia, CA). The quality and the quantity of the RNA were assessed by measuring the absorbance ratio of RNA at optical density (OD) values at 260 nm and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>) and OD<sub>260</sub> value respectively by NanoDrop (Thermo Scientific, Wilmington, DE). One microgram of total cellular RNA was brought to a volume of 41.5 μl using nuclease free water (Qiagen, Valencia, CA) and reverse transcribed in a 80 μl total volume reaction using 38.5 ul reverse transcription master mix containing 0.5 μl [20 U/μl] avian myeloblastosis virus [AMV] reverse transcriptase [Promega, Madison, WI], 1 μl oligo dT primer [0.5 μg/μl, Promega], 1 μl Rnasin plus RNase inhibitor [40 U/μl, Promega], 4 μl dNTP [10 mM, Promega], 16 μl AMV buffer [Promega], and 16 μl MgCl₂ [25 mM, Promega]) as described by Coombs et al. [344]. The reaction mixture was incubated at 42 °C for 15 min followed by 95 °C for 5 min in a thermocycler. The cDNA was then diluted 1:1 with sterile nuclease-free water (Qiagen, Valencia, CA). Equal amounts of cDNA were used for the gene expression analysis of different cytokines by TaqMan real-time PCR using an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The sequences of specific primers and probes used in this study targeting specific genes are provided in Table 2.3. In each PCR reaction in 384-well plates, 5 μl master mix (SensiMix™ II Probe Kit, Bioline), 0.5 μl 20X primer-probe mix (Applied Biosystems) and 4.5 μl template cDNA were added and incubated at 95 °C for 10 min, followed by 40 cycles for 15 sec each, and 60 °C for 1 min.
Table 2.3: Primers and probes for real-time RT-PCR used in this study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequences (5’ to 3’)/ Information</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EqRPLP0</td>
<td>Fwd: CTGATTACACCTTCCACTTGCT &lt;br&gt; Rev: AGCCACAAATGCAGATGGATCA &lt;br&gt; Probe: FAM-AAGG CCTTGACCTTTTC-NFQ</td>
<td>Boliar et al [345]</td>
</tr>
<tr>
<td>EqIFN-β</td>
<td>5’-AATGGCCCTCTCTGCTGTG-3’ &lt;br&gt; 5’-CCGAAGCAAGTCATAGTTACAGA-3’ &lt;br&gt; FAM-CTCCACCACGGCTC-NFQ</td>
<td>Horohov et al [346]</td>
</tr>
<tr>
<td>EqISG56</td>
<td>Fwd: AGCCCATTCAGCTAAACAAAAAC &lt;br&gt; Rev: CAGTTGCTTCAATTAGCAAATCCC &lt;br&gt; Probe: 5’- /56-FAM/TCC AGC CTG /ZEN/TCC TTG ATG TGA TCTTC/3IABkFQ/ -3’</td>
<td>Sarkar et al from this study</td>
</tr>
<tr>
<td>EqViperin</td>
<td>Fwd: CGCCAGTGCAATTACAAGTG &lt;br&gt; Rev: TCTCCGCCCCGAAAGTTTATC &lt;br&gt; Probe: 5’- /56-FAM/CTT CCA CAC /ZEN/GGC CAA GAC TTCCT/3IABkFQ/ -3’</td>
<td>Sarkar et al from this study</td>
</tr>
</tbody>
</table>

All reactions were performed in duplicate. The quantitative real-time PCR (qPCR) data were normalized to mRNA levels of equine ribosomal protein large P0 (RPLP0) as endogenous controls. Initially, several house-keeping genes [RPLPO, β glucuronidase (β-GUS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin, β-actin and eukaryotic 18s rRNA] were tested and the expression level of RPLPO was found to be the most stable during EHV-1 infection. As a result, RPLPO was used to normalize the qPCR data. PCR efficiency for all reactions was assessed by LinReg software [347]. Fold changes in the gene expression were calculated by the relative quantitation method described by Livak et al [348] which assumes that the relative concentration of target mRNA is equal to $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = [(\text{Avg. Ct of gene of interest} – \text{Avg. Ct of RPLP0)}_{\text{sample}} – (\text{Avg. Ct of gene of interest} – \text{Avg. Ct of RPLP0})_{\text{calibrator}}]$. The average $\Delta Ct$ value of mock infected samples for each individual gene was used as the calibrator.
2.9 Type-I Interferon Bioassay

For interferon bioassays, EECs were either mock infected or EHV-1 infected or treated as specified and the supernatants were harvested at different times post infection. The supernatants were filtered through Vivaspin 2 (Sartorius Stedim North America Inc., Bohemia, NY) to remove virus particles. Filtered supernatants were added on to a monolayer of MDBK cells at 100 μl/well in duplicate wells and incubated for 24 h at 37°C, 5% CO₂. The efficiency of the Vivaspin 2 filter to remove virus particles from TCF was confirmed by the absence of CPE when looked under an inverted light microscope. MDBK cells were then grown in 96-well plates and at 80% confluency the cells were treated with 100 μl/well of serially two fold diluted filtered supernatants. After 24 h of incubation, the cells were washed twice with warm D-PBS and challenged with VSV-GFP at an MOI of 0.1. At 18 h post challenge, the growth medium was discarded and cells were then stained with 1% crystal violet with buffered formalin. The results were expressed as laboratory unit (LU)/ml. The dilution in which 50% of a well was protected after VSV-GFP challenge was considered to have one LU of type-I interferons.

2.10 Immunofluorescence Assay

EECs were grown on poly L-lysine-coated coverslips (Fisher Scientific, Waltham, MA) in 24-well plates and treated as specified. At indicated time points, the cells were washed with ice cold PBS (pH 7.4) and fixed with 4% PFA at room temperature (RT) for 30 min. After washing 3X with PBS-glycine (10mM glycine in PBS), cells were permeabilized with 0.1% Triton X-100 in D-PBS (pH 7.4) for 10 min at RT, washed again 3X times with PBS-glycine (10 mM) and then blocked with 5% goat serum in PBS for 30 min at RT. The coverslips were then washed again 3X with PBS-glycine and incubated with primary antibodies at RT for 50 min. The name of the antibodies and their dilutions used in this study are enlisted in table 2.1. Next, the unbound primary antibodies were washed off with PBS-glycine and then the coverslips were incubated with corresponding secondary antibodies conjugated with either fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) for 50 min at RT. Finally, the
coverslips were washed and mounted in VECTASHIELD Mounting Medium containing 4’, 6’-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and observed under an inverted fluorescence microscope (Nikon ECLIPSE Ti, Melville, NY, USA).

2.11 Western Blot Analysis

EECs were grown on 6-well plates and treated with T953 or as described. At indicated time points the cells were washed in cold PBS (pH 7.4) and harvested in cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA; Sigma), 1% Triton X-100 (Sigma), 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF; Fisher Scientific) enriched with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktails, Thermo Scientific, Rockford, IL) on ice. The lysates were briefly sonicated and centrifuged at 14,000 g for 10 min at 4 °C. These protein samples were mixed with 5X reducing sample buffer (Lane Marker Reducing Sample Buffer, Thermo Scientific,) containing proprietary pink tracking dye in 0.3M Tris-HCl, 5% sodium dodecyl sulfate (SDS), 50% glycerol, 100mM dithiothreitol (DTT), boiled at 95 °C for 5 min and cooled immediately. Denatured proteins were separated onto 10 % SDS polyacrylamide resolving gels and 5 % stacking gels at 200 V for 40 min. After that, proteins were transferred onto a polyvinylidene difluoride (PVDF, Bio-Rad Laboratories, CA) membranes at 100 V for 50 min to 75 min (depending upon the molecular weight of the protein of interest) and blocked with 5 % non-fat dry milk (Bio-Rad) in Tris-buffered saline (TBST) for 1 h at RT. Following blocking, the membranes were incubated overnight with primary antibodies either in blocking buffer or 5 % bovine serum albumin (BSA) in TBST at 4 °C. The unbound primary antibodies were removed by washing the membranes 3X with TBST, with 10 min each washing. The membranes were then incubated with corresponding secondary antibodies for 1 h at RT followed by 3X washing with TBST (10 min each wash) and then developed by using the enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate Kit, Thermo Scientific) detection method.
The protein bands in the membrane were visualized by Fluorche E System (Cell Biosciences, San Jose, CA).

2.12 Separation of Cytoplasmic and Nuclear Proteins

EECs were maintained in T150 flasks in complete growth media and infected with T953 at an MOI of 5 or left uninfected. At indicated time points the cells were lysed and cytoplasmic as well as nuclear proteins were separated by cell fractionation by NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific, Rockford, IL, USA) as per manufacturer’s protocol. Briefly the cells were washed two times with D-PBS (pH 7.4) and harvested with trypsin-EDTA and centrifuged at 500 x g for 5 min. The pellet was washed again by suspending it in D-PBS and pelleted again by centrifugation at 500 x g for 3 min. The supernatants were removed very carefully leaving the pellet as dry as possible. Next, an appropriate volume of cytoplasmic extraction reagent I (CER I) solution was added to resuspend the pellet with vigorous vortexing. The volume of CER I was dependent on the volume of cell pellet following the chart supplied with the kit. The tube was incubated on ice for 10 min and then ice cold CER II was added followed by vortexing. It was then incubated on ice for 1 min and vortexed again and centrifuged at 16000 x g for 5 min at 4 °C. Immediately after that the supernatant containing the cytoplasmic proteins were transferred into pre-chilled tubes and stored at -80 °C until further use. The insoluble pellet containing nuclei was resuspended again using ice cold nuclear extraction reagent (NER) by vortexing briefly and was incubated on ice for 40 min with 15 seconds vortexing at 10 min intervals. The tubes were then centrifuged at 16000 x g for 10 min at 4 °C and the supernatant containing the nuclear proteins was separated and stored at -80 °C. The cytoplasmic and nuclear protein lysates were mixed with 5X sample buffer for separation by SDS-PAGE in a 10% bis-crosslinked polyacrylamide gel as described earlier. After separation, proteins were transferred onto a PVDF membrane and blotted with corresponding primary and secondary antibodies and developed as described earlier.
2.13 Quantification of Band Intensity
The relative intensity of the expression level of different protein bands in the western blot images was quantified by densitometric analysis using ImageJ software, National Institute of Health (NIH), USA following the manufacturer’s instructions. The

2.14 Statistical Analysis
All the experiments were repeated 3 times if not otherwise stated. The data were analyzed either by Student’s t test or analysis of variance (ANOVA) and Pairwise Multiple Comparison Procedures by Holm-Sidak Method using statistical analysis software Sigmaplot 12.3 (Systat Software Inc. San Jose, CA). In the case when only two groups were compared (as in the case of real-time PCR data in ISG mRNA induction experiment), Student’s t test was performed. In all other cases, data were analyzed by ANOVA. P values of less than 0.05 % were considered as statistically significant.

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CHAPTER III: RESULTS

3.1 Standardization of EHV-1 Infection in EECs

To determine the MOI to be used for the study, EECs were grown on 6-well plates and were infected with a neuropathogenic strain of EHV-1, T953 at an MOI of either 0.5, 5 or 10. At 12 h post infection, cells were fixed with 4% PFA and the virus infection was evaluated by IFA using EHV-1 IE1 specific antibody. It was found that >95% cells were IE1 positive at an MOI of 5 (Figure 3.1) and hence, this MOI was selected, unless otherwise stated.

Figure 3.1: Immunofluorescence images showing the infection of EECs with T953: EECs were infected with T953 at an MOI of 5 and at 12 h.p.i. the cells were fixed and stained with anti-rabbit IE1 antibody. Mock treated EECs stained with DAPI for nucleus (A), anti-rabbit IE1 Ab conjugated with FITC (B), and merged images of A and B (C). T953 infected EECs stained with DAPI (D), anti-rabbit IE1 Ab conjugated with FITC (E), and merged images of D and E (F).
3.2 Replication of T953 in EECs:

To evaluate whether T953 can replicate and produce infectious progeny virus in EECs, monolayers of EECs were infected at an MOI of 5 and virus titers in the supernatants of infected cells collected at various time post-infection (3, 6, 12 and 18 h.p.i.) were determined by plaque assay. Results showed that T953 can successfully replicate in the EECs producing progeny virus.

![Graph showing replication of T953 in EECs](image)

**Figure 3.2: Replication of T953 in EECs:** EECs were infected with T953 at an MOI of 5 and at 3, 6, 12 and 18 h.p.i. supernatants from the infected cells were collected and titrated for virus. At 18 h.p.i. highest titer of the virus were seen. Data represents mean with standard deviation from 3 independent experiments.
3.3 Effect of T953 Virus Infection on IFN-β Gene Transcription in EECs

T953, a neuropathogenic strain of EHV-1, is an endotheliotropic virus which replicates in the endothelial cells of blood vessels, causing ischemic damage which results in the neurologic disease, EHM. One of the sub-hypotheses was that T953 virus suppresses type-I IFN induction in the infected endothelial cells making the microenvironment more conducive for enhanced viral replication which adds to the severity of the disease. Previously, it has been shown that EHV-1 infections of PBMCs induce significant amounts of type-I IFN both in vivo and in vitro [332, 349], but there is no report whether EHV-1 has the ability to interfere with type-I IFN immune responses of horse. Here, EECs were infected with T953 at an MOI of 5. SeV and poly I:C served as positive controls for the type-I IFN induction. Virus diluent (DMEM + 2% FBS) treated EECs were used as negative controls. At 3, 6, 12 and 18 h.p.i. IFN-β gene expression was evaluated by real-time RT-PCR assay. T953 infection of EECs induced a significant amount of IFN-β mRNA transcription (p<0.001) at early times post infection (3 and 6 h.p.i.) [Fig 3.3A] but at later time points (12 and 18 h.p.i.) there was no significant amount of IFN-β mRNA induction (Fig 3.3A). There was also a gradual decrease in the transcription of the IFN-β gene from 3 to 6 h.p.i. On the other hand, UV inactivated T953 virus did not induce any IFN-β mRNA either at early or late stage of the infections (Figure 3.3A).
Figure 3.3: T953 suppresses IFN-β mRNA induction. EECs were either mock infected or infected with different viruses separately or together or treated with chemicals as indicated. At indicated time points equine IFN-β mRNA was quantified by real-time RT-PCR and data were normalized to endogenous control equine RPLP0. T953 infection inhibited IFN-β mRNA at later time points (12 h onwards) after an initial induction at early time points (3 h) [Fig 3.3A]. T953 virus also suppressed IFN-β mRNA induced by SeV [Fig 3.3B] and poly I:C [Fig 3.3C]. Data represents mean with standard deviation from 3 independent experiments. *** = p<0.001, ** = p<0.01 and * = p<0.05 were considered significant.

To identify if T953 virus can inhibit the IFN-β mRNA induction by other known stimuli, EECs were coinfected with SeV at an MOI of 5 or co-treated with poly I:C and T953 virus for indicated time periods. It was observed that from 6 h.p.i. onwards, T953 and SeV coinfected EECs had significant decreases (p<0.01, p<0.001, p<0.001 at 6, 12 and 18 h.p.i respectively) in the IFN-β mRNA induction compared to only SeV infected EECs (Fig 3.3B). T953 virus infection also significantly suppressed the poly I:C induced IFN-β mRNA transcription at 12 h.p.i onwards (p<0.001) [Fig 3.3 C].
3.4 Effect of T953 Virus Infection on Type-I IFN Production in EECs

To evaluate the effect of T953 virus on type-I IFN induction at the translational level, a type-I IFN bioassay using a recombinant VSV-GFP virus was performed on the saved supernatants from the previously infected EECs for real-time RT-PCR assay. It was seen that T953 infection of EECs induced a significant amount of type-I IFN activity at 3 and 6 h.p.i. when compared to mock infected EECs (p<0.05) [Fig 3.4 A]. The virus infection also induced type-I IFN activity in the later time points (12 and 18 h.p.i.) [Fig 3.4 A], although there was a gradual decrease in the type-I IFN activity. As with IFN-β mRNA transcription, UV-inactivated T953 virus did not induce any significant amount of type-I IFN activity.

![Graph showing type-I IFN activity](image)
**Figure 3.4: T953 suppresses type-I IFN activity.** EECs were either mock infected or infected with different viruses separately or together or treated with chemicals as indicated. At indicated time points cell supernatants were collected and type-I IFN activity was quantitated by interferon bioassay using VSV-GFP. Type-I IFN bioassay is performed by incubating IFN-sensitive cells with serially diluted samples containing IFN and then challenging those cells with IFN-sensitive virus VSV-GFP. The well of a 96 well plate at which 50% of the cells were protected from VSV-GFP CPE was considered as 1 laboratory unit (LU). Data represents mean with standard error from 3 independent experiments. ** = p<0.01 and * = p<0.05 were considered significant. T953 infection to EECs induced type-I IFN activity at all time points (Fig 3.4A). But, T953 inhibited type-I IFN activity induced by SeV at 6 h, 12 h and 18 h (Fig 3.4B). The virus also suppressed poly I:C induced type-I IFN activity at 12 h and 18 h (Fig 3.4C).
To determine whether T953 virus could inhibit production of type-I IFN induced by other known stimuli at translational level, EECs were coinfected with T953 and SeV or T953 and poly I:C for different periods of time. It was observed that T953 coinfected with SeV induced significantly decreased type-I IFN activity at 6 h.p.i (p<0.05), 12 h.p.i. (p<0.01) and 18 h.p.i (p<0.01) compared to only SeV induced type-I IFN activity (Fig 3.4B). Data also suggested that T953 virus could suppress the type-I IFN activity induced by poly I:C significantly (p<0.05) at later times post infection (12 and 18 h.p.i.) [Fig 3.4C]. The results of these studies involving IFN-β gene expression kinetics also suggested that the longer the virus was incubated the more was the IFN-inhibitory effect. This suggested that expression of late viral genes might be responsible for the suppression of type-I IFN production.

3.5 The T953 Strain of EHV-1 Actively Suppresses IFN-β Gene Expression Induced by Sendai Virus

In the coinfection model of the previous experiment, EECs were infected with SeV and T953 together and were incubated for same period of time. It was possible that decreased IFN-β induction at later time points were due to the normal IFN kinetics or due to the absence of stimuli at late stage of the infection. To assure that T953 virus but no other reasons can actively suppress the type-I IFN production, a coinfection study was done where first T953 or SeV was allowed to express its genes for some time (3 h), then the same cells were infected with the other virus and were incubated for another 6 h. At 9th hour after first infection, IFN-β mRNA production was compared between coinfected EECs and EECs infected with only SeV.
Figure 3.5: T953 actively suppresses SeV induced INF-β mRNA transcription. EECs were either infected with SeV or T953 and at 3 h.p.i. cells were re-infected with T953 or SeV respectively for another 6 h. At 9 h.p.i. cells were lysed for total cellular RNA and IFN-β mRNA were quantitated from the reverse transcribed cDNA by real-time qPCR assay. Data were normalized to endogenous control equine RPLP0. Data represents mean with standard deviation from 3 independent experiments. T953 infection to EECs actively suppressed the transcription of IFN-β mRNA induced by SeV. Data were significant at p<0.001 (**).

When EECs were first infected with T953 virus and allowed to express its genes for 3 h, then infected the same cells with SeV for another 6 h, it was found that coinfected EECs produced a significantly lower amount of IFN-β mRNA (p<0.001) compared to only SeV infected cells for 6 h (Fig 3.5). Even when EECs were first stimulated with SeV for 3 h and then same cells were infected with T953 for another 6 h, there was a significant (p<0.001) decrease of IFN-β mRNA compared to only SeV infected cells for 9 h (Fig 3.5). These data suggested that T953 virus actively suppressed the type-I IFN induction capability induced by another known virus stimulus (SeV).
actively suppressed the type-I IFN induction capability induced by another known virus stimulus (SeV).

3.6 T953 Virus Actively Suppresses IFN-β Gene Expression Chemically Induced by Poly I:C

In the above coinfection experiment, it was considered possible that one virus infection to a cell could restrict the entry of another virus into the same cell. If so, it was possible that decreased IFN-β mRNA production by Sev and T953 coninfected EECs was due to the interference of SeV entry into the coinfected EECs by T953. To rule out that explanation for the suppression of IFN-β mRNA induction, EECs were first infected with T953 virus and allowed to express viral genes for different time periods (0, 3, 6 and 12 h) and then same infected cells were stimulated with the chemical inducer, poly I:C at 80 μg/ml for 12 h. IFN-β mRNA transcription was evaluated by real-time RT-PCR assay. The results showed that T953 infected EECs produced significantly lower amount of IFN-β mRNA when co-stimulated with poly I:C compared to only poly I:C treated cells (Fig 3.6) in all time points.
Figure 3.6: T953 actively inhibits poly I:C induced IFN-β mRNA transcription. EECs were infected with T953 virus and at 0, 3, 6 and 12 h.p.i. infected cells were stimulated with poly I:C at 80 μg/ml for an additional 12 h. Cells were then lysed for total cellular RNA which was reverse transcribed and IFN-β mRNA transcripts were quantitated by real-time qPCR assays. Equine RPLP0 was used as endogenous control to normalize the data. Data represents mean with standard deviation from 3 independent experiments. IFN-β mRNA transcription of T953 + poly I:C coinfected EECs was compared with only poly I:C treated cells. T953 actively inhibited transcription of chemically induced IFN-β mRNA by poly I:C. a, b, c are significantly different from each other at p<0.001.

From these data it was also evident that the longer the virus was allowed to express its genes, the greater the suppressive effect. By 12 h.p.i., T953 infected EECs stimulated with poly I:C were no longer able to make significant amounts of IFN-β mRNA. This supports the hypothesis that T953 can actively suppress type-I IFN induction by known exogenous stimuli including poly I:C and SeV.
can actively suppress type-I IFN induction by known exogenous stimuli including poly I:C and SeV.

3.7 Effect of Late Gene Blocking of EHV-1 on Type-I IFN Production

The preceding results show that T953 infection suppresses IFN-β responses and indicated a possible link of EHV-1 late genes with the reduced IFN-β mRNA at later time points. The next goal of this study was to block the late gene expression of T953 virus. EECs were infected with T953 virus in presence of a chemical inhibitor, phosphonoacetic acid (PAA) which is known to inactivate the herpes viral DNA polymerase [350]. The late gene expression of T953 virus at protein level was evaluated at 18 h.p.i. by an immunofluorescence study using a monoclonal antibody against the late gene, gC. A titration on PAA concentration was done to find the right concentration to be used without host cell toxicity. PAA at a concentration of 300 μg/ml of growth media during T953 infection to EECs, prevented the majority of viral late gene, gC expression (Fig.3.7). In addition to blocking of L (γ) genes, it was also evaluated whether PAA had any effect on IE gene expression by using another antibody (α-IE1 Ab) specific to IR1 protein (IE gene product). The results showed that IE gene expression was unaffected by PAA treatment (Fig 3.7). This confirms that PAA blocks the late gene expression of herpesvirus including EHV-1 but it does not interfere with the expression of IE gene and thus allowed for the evaluation of the effect of IE (α) and E (β) genes on the suppression of the type-I IFN both at mRNA and protein levels. EECs were infected with T953 in the presence or absence of PAA for 3, 6, 12 and 18 h. EECs treated only with PAA in virus diluent medium were used as negative controls. At indicated time points type-I IFN production was evaluated by measuring the transcription of IFN-β mRNA by real-time RT-PCR assay as well as type-I IFN activity by interferon bioassay.
Figure 3.7: PAA blocks late gene expression but not IE gene expression. EECs were infected with T953 in the presence or absence of PAA at 300 μg/ml. At 18 h.p.i. viral gene expression was evaluated by IFA using EHV-1 late gene gC specific Ab (anti-gC Ab) and IE gene specific Ab (anti-IE1 Ab). Upper panel shows the staining of mock infected EECs treated with PAA. Middle panel shows that in the presence of PAA, T953 infected EECs showed no gC expression but showed IE gene expression. The lower panel shows that in the absence of PAA, T953 infected EECs showed expression of both the late gene, gC and the sole IE gene.
**Figure 3.8 A: Late gene blocking induces robust IFN-β mRNA transcription at later time points.** EECs were infected with T953 in the presence or absence of PAA and at indicated time points cells were harvested to extract total cellular RNA. Transcription of IFN-β mRNA was quantitated by real-time RT-PCR assay. Data were normalized to the endogenous control equine RPLP0. Data represents mean with standard deviation from 3 independent experiments. Late gene blocking of T953 by use of PAA induced more IFN-β mRNA in EECs compared to only T953 infected EECs without PAA at 12 and 18 h.p.i. Data are significantly different from each other at p value of <0.01 (**).

Significant differences (p<0.01) were observed in the IFN-β mRNA transcription starting at 12 h.p.i. and at 18 h.p.i. T953 infected EECs in the presence of PAA caused a robust IFN-β mRNA production compared to T953 infected EECs in the absence of PAA (Fig. 3.8 A). It was further investigated whether blocking of viral late genes had the same type-I IFN inducing effect at the level of translation. Results of the type-I IFN bioassay performed on the infected cell supernatants showed that T953 virus infected EECs produced significantly higher levels of type-I IFN activity at 12 and 18 h.p.i. when expression of viral late genes was blocked by PAA treatment compared
to T953 infected EECs in the absence of PAA treatment (p<0.05 and p<0.001, Fig 3.8 B). It is interesting to note that the same effect was even more evident at 24 h.p.i. (data not shown).

**Figure 3.8 B: Late gene blocking induces robust type-I IFN activity at later time points.**

EECs were infected with T953 in the presence or absence of PAA and at indicated time points cell supernatants were collected and titrated for type-I IFNs by interferon bioassay. Data represents mean with standard deviation from 3 independent experiments. Late gene blocking of T953 by use of PAA induced significantly more type-I IFN activity in EECs compared to only T953 infected EECs at 12 and 18 h.p.i. Data were considered significant at p value of <0.01 (**) and p<0.05 (*).

These data suggest that EHV-1 has mechanisms which actively inhibit type-I IFN immune responses and viral late genes but not IE or E genes play a major role in this suppression.
3.8 Effect of T953 Virus Infection on the Stability of IRF-3

It is well established that for the production of type-I IFN, triggering of either IRF-3/7, AP-1 or NF-κB transcription factors is necessary. As reviewed above, different viruses may target different transcription factors to interfere with production of this important host antiviral cytokine. Like BHV-1 IE protein bICP0 or HSV-1 it seemed plausible that EHV-1 protein(s) directly degrade the most important transcription factor, IRF-3 in the type-I IFN production pathways. To elucidate the mechanisms, EECs were either infected with T953 or mock infected. At 3, 6, and 12 h.p.i. cells were lysed to prepare total cell lysates and were analyzed by Western blot using anti-IRF-3 antibody.

The Western blot analyses on T953 infected cell lysates showed that virus infection of EECs indeed caused down-regulation of the endogenous level of IRF-3 proteins (Fig 3.9 A) as early as at 6 h.p.i. At 12 h.p.i. the majority of the endogenous IRF-3 level was down-regulated when compared to mock infected cell lysates. Western blot image also suggested that the intensity of IRF-3 down-regulation increased with the increased expression of viral late gene, gC (Fig 3.9 C). Densitometric analyses of the Western blot images suggested about 3.4 X and 105 X decreased level of total endogenous IRF-3 protein in the T953 infected EECs at 6 and 12 h.p.i. respectively when compared to mock infected EECs (Fig 3.9 D).
**Figure 3.9: T953 causes down-regulation of IRF-3.** EECs were infected with either T953 or mock infected. At indicated time points whole-cell lysates were prepared and equal amounts (40 μg) of lysates were separated by 10% SDS-PAGE and blotted with (A) anti-IRF-3, (B) anti-β-actin and (C) EHV-1 late protein gC antibodies. β-actin was used as endogenous loading control. (D) Densitometric analyses were done by NIH ImageJ software and the IRF-3 band intensity was normalized to β-actin band intensity. T953 infected cell lysates showed a decrease in relative fold intensity of IRF-3 compared to mock infected cells. The Western blot images were representative of 3 independent experiments. T953 induced down-regulation of IRF-3 at later time points (6 h and 12 h) in EECs when compared to mock infected EECs. Level of IRF-3 protein was reduced with the increase in late protein gC.
3.9 Role of T953 Virus in the Activation of IRF-3

It was evident from results of our previous experiments that, among possible mechanisms for interference with host type-I IFN production pathways, T953 directly or indirectly targeted transcription factor IRF-3 for its down-regulation. It was however, important to know if T953 virus also interfered with other steps in the activation of IRF-3 signaling pathways including phosphorylation of IRF-3 or nuclear translocation of phosphorylated IRF-3 (pIRF-3) from cytoplasm. Therefore, EECs were either mock infected or infected with T953. At 3 and 12 h.p.i., cells were lysed and nuclear and cytoplasmic fractions of total proteins were separated by subcellular fractionation. SeV infection of EECs for 12 h was considered as positive control. Protein fractions were analyzed by Western blot using anti-pIRF-3, anti-IRF-3 and anti-lamin A/C Abs.

Western blot analyses (Fig 3.10) of the subcellular protein fractions of infected cell lysates showed that total (cytoplasmic plus nuclear) level of pIRF-3 in T953 infected EECs was down-regulated from early (3 h) time points to later (12 h) time points. It was also evident that at 3 h T953 infection induced activation of IRF-3 molecules by phosphorylation and majority of the pIRF-3 was translocated into the nucleus from the cytoplasm (Fig 3.10 A). On the other hand, at 12 h.p.i. the virus induced phosphorylation was greatly reduced and there was down-regulation in the nuclear accumulation of the activated pIRF-3 when compared with 3 h.p.i. (Fig 3.10 A). When membranes were blotted with anti-IRF-3 Abs which detected total IRF-3 (both phosphorylated as well as unphosphorylated IRF-3) it was also observed that at early time points, the majority of the total IRF-3 molecules were translocated into the nucleus when compared with cytoplasmic IRF-3 (Fig 3.10 B, C). At later time points, however, the majority of the total IRF-3 molecules were in the cytoplasm (Fig 3.10 B, C). Since, IRF-3 molecules in general translocate into the nucleus only after activation by phosphorylation, the down-regulation in the amount of total nuclear IRF-3 at later time points also indirectly suggests a down-regulation in the activation of IRF-3 molecules at those time points. It was noted that IRF-3 Ab (FL-425) from Santa Cruz
Biotechnology detected a nonspecific band in the mock infected nuclear lysates which was not detected when membranes were blotted with IRF-3 mAb (D6I4C) from Cell Signaling Technology (Fig 3.10 C). Densitometric analyses of the Western blot images showed that the fraction of total nuclear IRF-3 that was phosphorylated (pIRF-3/IRF-3) decreased over time when compared between nuclear lysates from 3 h.p.i. and 12 h.p.i. (Fig 3.10 J). Interestingly, the ratio of pIRF-3/IRF-3 in the cytoplasmic lysates increased over time when compared between 3 h.p.i. and 12 h.p.i. This increased cytoplasmic pIRF-3 fraction at later time points suggested that T953 infection either prevented further translocation of pIRF-3 into the nucleus from cytoplasm at 12 h.p.i. or the virus infection triggered nuclear pIRF-3 to translocate back into cytoplasm. In both cases, however, there was a down-regulation in the activation of IRF-3 at later time points. The data suggest that the transcription factor IRF-3 gets normally activated and translocated into the nucleus of the infected cells at early time points but at later time points IRF-3 signaling pathways are blocked either by inhibiting the translocation of pIRF-3 from cytoplasm to nucleus or stimulating pIRF-3 to translocate back into the cytoplasm from the nucleus or preventing IFR-3 phosphorylation by degrading endogenous IRF-3 making IRF-3 unavailable for phosphorylation.
**Figure 3.10: T953 prevents activation of IRF-3.** EECs were infected with either T953 or mock infected. At indicated time points cells were lysed and nuclear and cytoplasmic protein lysates were separated. Equal amounts (20 μg) of lysates were separated by 10% SDS-PAGE and blotted with (A) anti-pIRF-3, (B, C) anti-IRF-3 and (D) anti-lamin A/C antibodies. Lamin A/C was used as nuclear loading control. Protein fractions of SeV infected cell lysates analyzed with (E) anti-pIRF-3, (F) anti-IRF-3 (FL-425) and (G) anti-lamin A/C Abs were used as positive control. (H-K) Densitometric analyses were done by NIH ImageJ software and the fraction of IRF-3 that was phosphorylated was determined by the ratio of pIRF-3 to IRF-3 band intensity. The Western blot images were representative of 2 independent experiments.
Figure 3.10: T953 prevents activation of IRF-3. EECs were infected with either T953 or mock infected. At indicated time points cells were lysed and nuclear and cytoplasmic protein lysates were separated. Equal amounts (20 μg) of lysates were separated by 10% SDS-PAGE and blotted with (A) anti-pIRF-3, (B, C) anti-IRF-3 and (D) anti-lamin A/C antibodies. Lamin A/C was used as nuclear loading control. Protein fractions of SeV infected cell lysates analyzed with (E) anti-pIRF-3, (F) anti-IRF-3 (FL-425) and (G) anti-lamin A/C Abs were used as positive control. (H-K) Densitometric analyses were done by NIH ImageJ software and the fraction of IRF-3 that was phosphorylated was determined by the ratio of pIRF-3 to IRF-3 band intensity. The Western blot images were representative of 2 independent experiments.

As reviewed earlier activation of IRF-3 by phosphorylation followed by translocation from cytoplasm to nucleus is a key step in the IRF-3 signaling pathways. Studies were designed to confirm whether T953 virus was able to block the IRF-3 activation pathways by preventing nuclear accumulation of IRF-3.
Indirect immunofluorescence images of EECs that were either mock infected or infected with T953 virus for 1, 3, 6 and 12 h. (Fig 3.11) clearly showed that in the mock infected EECs at all time points (1, 3, 6, and 12 h.p.i.) the transcription factor IRF-3 was mainly cytoplasmic (Fig 3.11.II, 3.11.III, 3.11.IV, 3.11V); but when activated by a known stimulus, poly I:C, the majority of the IRF-3 molecules translocated into the nucleus (Fig 3.11.I).

Figure 3.11.I: Poly I:C induces nuclear accumulation of IRF-3. EECs were stimulated with poly I:C and at indicated time points cells were fixed in 4% PFA and analyzed by IFA. Panels show EECs that were poly I:C treated for 3 h were stained with anti-IRF-3 and anti-EHV-1 gC Abs. (A) Nuclear stain DAPI (blue), (B) IRF-3 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified cells. Poly I:C stimulated EECs showed nuclear localization of IRF-3.
**Figure 3.11.II: T953 prevents nuclear accumulation of IRF-3.** EECs were infected with T953 or mock infected and at indicated time points cells were fixed in 4% PFA and analyzed by IFA. Panels show EECs that were mock infected for 1 h (upper panel) and T953 infected for 1 h (lower panel) were stained with anti-IRF-3 and anti-EHV-1 gC Abs. (A) Nuclear stain DAPI (blue), (B) IRF-3 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified cells. T953 infected EECs showed both cytoplasmic and nuclear IRF-3 while mock infected cells showed cytoplasmic IRF-3.
Figure 3.11.III: T953 prevents nuclear accumulation of IRF-3. EECs were infected with T953 or mock infected and at indicated time points cells were fixed in 4% PFA and analyzed by IFA. Panels show EECs that were mock infected for 3 h (upper panel) and T953 infected for 3 h (lower panel) were stained with anti-IRF-3 and anti-EHV-1 gC Abs. (A) Nuclear stain DAPI (blue), (B) IRF-3 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified cells. Majority of the T953 infected EECs showed nuclear IRF-3 while mock infected cells showed cytoplasmic IRF-3.
**Figure 3.11.1V: T953 prevents nuclear accumulation of IRF-3.** EECs were infected with T953 or mock infected and at indicated time points cells were fixed in 4% PFA and analyzed by IFA. Panels show EECs that were mock infected (upper panel) and T953 infected for 6 h (lower panel) were stained with anti-IRF-3 and anti-EHV-1 gC Abs. (A) Nuclear stain DAPI (blue), (B) IRF-3 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified cells. T953 infected EECs showed both cytoplasmic and nuclear IRF-3 while mock infected cells showed cytoplasmic IRF-3.
**Figure 3.11.V: T953 prevents nuclear accumulation of IRF-3.** EECs were infected with T953 or mock infected and at indicated time points cells were fixed in 4% PFA and analyzed by IFA. Panels show EECs that were mock infected (upper panel) and T953 infected for 12 h (lower panel) were stained with anti-IRF-3 and anti-EHV-1 gC Abs. (A) Nuclear stain DAPI (blue), (B) IRF-3 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified cells. The majority of the IRF-3 molecules were cytoplasmic both in T953 as well as mock infected EECs.
In the T953 infected EECs, IRF-3 molecules started to become activated and translocated from cytoplasm into nucleus as early as 1 h.p.i. when the majority of IRF-3 molecules were still in the cytoplasm (Fig 3.11.II). At 3 h.p.i., however, the majority of the IRF-3 molecules were found to have translocated from the cytoplasm and accumulated in the nucleus (Fig 3.11.III). At 6 h.p.i. IRF-3 was found both in the cytoplasm as well as in the nucleus of virus infected EECs although the majority of the IRF-3 molecules were still in the nucleus (Fig 3.11.IV). At 12 h.p.i. the majority of the IRF-3 molecules were in the cytoplasm of the T953 infected EECs (Fig 3.11.V).

These data supported the findings of the Western blot images (3.10) that after an initial activation followed by nuclear accumulation of IRF-3 at early times post infection, T953 virus induced a gradual decrease in the nuclear accumulation of activated IRF-3 in EECs in vitro which could be responsible for suppressing type-I IFN production at later times.

3.10 Effect of T953 Infection on NF-κB Signaling Pathways

In addition to the IRF-3 signaling pathways, NF-κB signaling pathways also play an important role in the production of type-I IFNs. To determine whether the virus was able to suppress the NF-κB mediated signaling pathways to inhibit type-I IFN induction, EECs were either infected with T953 or mock infected. At 3, 6, and 12 h.p.i., cells were lysed for total cellular lysates and were analyzed by Western blot with anti-IκBα, anti-β-actin and anti-IE1 Abs. Normally in the absence of stimuli, NF-κB transcription factors remains sequestered by associating with another inhibitory protein IκBα in the cytoplasm. When induced by a stimulus, IκBα is phosphorylated and degraded by cellular proteasome and subunits of NF-κB are released and activated [351]. These activated NF-κB subunits then enter into the nucleus and bind to the DNA of the cell regulating the transcription of a number of genes including IFN-β (Fig 1.8). The results showed that T953 infection caused a down-regulation of the endogenous level of NF-κB inhibitory protein, IκBα (Fig 3.12.I) suggesting an activation of the NF-κB signaling pathways at later time points (12 h.p.i.).
Figure 3.12.I: T953 infection induces degradation of IκBα. EECs were infected with either T953 or mock infected. At indicated time points whole-cell lysates were prepared and equal amounts (40 μg) of lysates were separated by 10% SDS-PAGE and blotted with (A) anti-IκBα, (B) anti-β-actin and (C) EHV-1 IE1 antibodies. T953 induced degradation of IκBα at later time points (12 h) in EECs when compared to mock infected EECs. (D) Densitometric analyses were done by NIH ImageJ software and IκBα band intensity was normalized to β-actin band intensity. T953 infected cell lysates showed a decrease in relative fold intensity of IκBα compared to mock infected cells. The Western blot images were representative of 3 independent experiments.

Additionally, NF-κB signaling pathways were also studied directly by IFA using NF-κB p50 specific Ab (Fig 3.12.II). EECs were either mock infected (negative control) or infected with T953 for indicated time points. Mock infected EECs stimulated with TNF-α at 45 ng/ml of media for 45 min were the positive control. Infected or TNF-α treated cells were then stained with NF-κB p50 specific primary Ab. It was observed that in mock infected cells in the absence of TNF-α treatment, the majority of the p50 subunit was located in the cytoplasm (Figure 3.12.II i, and 3.12.II.ii).
Figure 3.12.Ii: T953 infection does not activate NF-κB signaling pathways at early time points. EECs were mock infected (upper panel) or infected with T953 (lower panel) for 3h. Cells were then fixed in 4% PFA and analyzed with IFA using anti-NF-κB p50 and anti-EHV-1 gC Abs. Figure shows (A) Nuclear stain DAPI (blue), (B) NF-κB p50 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified field. Majority of the NF-κB p50 molecules were cytoplasmic both in T953 as well as mock infected EECs.
Figure 3.12.Ii: T953 infection activates NF-κB signaling pathways at later time points.

EECs were mock infected (upper panel) or infected with T953 (lower panel) for 12h. Cells were then fixed in 4% PFA and analyzed with IFA using anti-NF-κB p50 and anti-EHV-1 gC Abs. Figure shows (A) Nuclear stain DAPI (blue), (B) NF-κB p50 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified field. In T953 infected EECs, the majority of the NF-κB p50 molecules were located in the nucleus while in mock infected cells, NF-κB p50 were in the cytoplasm.
Figure 3.12.I.iii: TNF-α causes activation of NF-κB p50 signaling pathways. EECs were stimulated with TNF-α at 45 ng/ml for 45 min. Then the treated cells were fixed in 4% PFA and analyzed with IFA using anti-NF-κB p50 and anti-EHV-1 gC Abs. Figure shows (A) Nuclear stain DAPI (blue), (B) NF-κB p50 (green), (C) EHV-1 gC (red) and (D) merged images. Long white arrows indicate magnified field. TNF-α stimulation caused nuclear translocation of NF-κB p50 suggesting an activation of NF-κB signaling pathways.

But in the mock infected EECs treated with TNF-α, the majority of the p50 subunit was translocated into the nucleus (Fig 3.12.I.iii). At early time points (3 h) after T953 infection, the majority of the cells showed p50 subunit located in the cytoplasm indicating that NF-κB signaling pathways were not activated (Fig 3.12.I.i). On the other hand, at later time points after T953 infection (12 h), the majority of the infected EECs were observed to possess p50 subunit in the nucleus (Fig 3.12.I.ii) indicating that NF-κB signaling pathways were activated. In summary, the results thus show that at the beginning of infection, NF-κB signaling pathways were not activated while at later time points of the virus infection the pathways were activated. These important findings also suggest that inhibition of type-I IFN by T953 infection in EECs at later time points was probably not due to the inhibition of NF-κB signaling pathways.

Altogether, the results shown in Figures 3.9, 3.10, 3.11 and 3.12 reveal that T953 virus inhibited type-I IFN production in EECs at later time points by a mechanism which includes
inhibiting IRF-3 signaling pathways through multiple strategies such as inducing degradation of IRF-3 and also preventing nuclear accumulation of IRF-3.

### 3.11 Effect of Exogenous rEqIFN-α on the Replication of T953

The previous results support that EHV-1 has mechanisms to inhibit type-I IFN response at the production level by interfering with IRF-3 signaling pathways. But it was not clear whether the virus can also interfere with the effect of type-I IFN as there were contradictory reports regarding the antiviral effect of type-I IFN against EHV-1. Therefore, it was important to determine the effect of type-I IFN on the replication of T953 virus *in vitro*. For this, EECs were pre-treated for 24 h either with rEqIFN-α at a concentration of 1000 IU/ml of growth media or with equal volume of MEM in growth media and then the cells were challenged with T953 at a low MOI (MOI=0.5). At indicated time points (3, 6, 12, 24, 36 and 48 h.p.i.) cell supernatants were collected and titrated for the presence of T953 virus by plaque assay (Fig 3.13). The results of the plaque assay showed that exogenous treatment of the rEqIFN-α had very little inhibitory effect (less than 10 fold) on T953 virus replication at 12 and 24 h.p.i. At 36 and 48 h.p.i. this inhibitory effect was even less. In all the time points, however, no statistically significant differences in T953 titers were found between the rEqIFN-α treated and untreated EECs (Fig 3.13).
Figure 3.13: rEqIFN-α cannot block the replication of T953 in EECs. Cells were pre-treated for 24 h with rEqIFN-α at 1000 IU/ml of media and were challenged with T953 at an MOI of 0.5. At indicated time points cell supernatants were collected and T953 virus titers were determined by plaque assay. Bars show the means with standard errors from 3 independent experiments. rEqIFN-α treatment did not inhibit T953 replication.

Figure 3.14: rEqIFN-α inhibits replication of VSV-GFP. (A) EECs were either pre-treated with rEqIFN-α at 1000 IU/ml of media or left untreated for 24 h and then challenged with VSV-GFP at an MOI of 0.5 for another 24 h. VSV-GFP replication was evaluated by determining the expression of GFP under inverted fluorescent microscope. (A) rEqIFN-α treatment completely inhibited expression of GFP (B) EECs untreated with rEqIFN-α could not suppress the expression of GFP. The images were representative of 3 independent experiments. rEqIFN-α treatment completely inhibited VSV-GFP replication.
As a positive control EECs were either pre-treated with rEqIFN-α for 24 h or left untreated and then challenged with an interferon sensitive virus, VSV-GFP at an MOI of 0.5. At 24 h.p.i. effect of rEqIFN-α on replication of the VSV-GFP virus was determined by evaluating the expression of GFP under an inverted fluorescent microscope. No GFP expression was observed in the VSV-GFP infected EECs pre-treated with rEqIFN-α at 1000 IU/ml of media (Fig 3.14 A). On the other hand, EECs which were not treated with rEqIFN-α showed expression of GFP (Fig 3.14 B). VSV is known to be a type-I IFN-sensitive virus. The inhibition of VSV-GFP replication by rEqIFN-α in EECs confirmed that VSV is IFN-sensitive and also confirmed that EECs were IFN-competent cells.

3.12 Effect of rEqIFN-α on the Infection Process of T953 Virus in EECs

In the previous experiment (Fig 3.13), although rEqIFN-α had a little inhibitory effect (not statistically significant) at 12 and 24 h.p.i., even this minor inhibitory effect was further reduced by 36 and 48 h.p.i. It was possible that rEqIFN-α treatment would induce antiviral proteins or ISGs and would make the majority of cells resistant to T953 virus infection but few cells were still susceptible. When those few cells were infected, they produced progeny viruses which eventually amplified in numbers by more rounds of replication. By 24 h.p.i. these amplified progeny viruses could overpower the cellular antiviral immunity induced by ISGs and infect more nearby uninfected cells. These caused increase in the titer of the virus by 48 h which were not different when compared with the virus titer from the IFN-untreated cells. Alternatively, it could also be possible that rEqIFN-α treatment would cause all the cells equally resistant or susceptible to T953 infection. When challenged with T953 virus, equal number of cells were infected from IFN-treated and untreated cells. It is possible that antiviral immunity in the IFN-treated cell might prolong the multi-cycle infection process of the virus till 24 h.p.i. but did not completely block it. As a result, in spite of an initial little difference between virus titers (12 h and 24 h) between IFN-
treated and untreated infected cells, there was no significant difference later (36 h and 48 h). To differentiate that, an infectious center assay was performed to elucidate whether IFN-treatment caused a difference in the number of infectious centers or a difference in the infected cell numbers or the IFN treatment prolonged the multi-cycle infection process.

**Figure 3.15: rEqIFN-α does not inhibit replication of T953 virus.** EECs were either pre-treated with rEqIFN-α at 1000 IU/ml of media or left untreated. At 24 h cells were infected with T953 at an MOI of 0.5. After the adsorption of virus at 37 °C for 1 h, cells were trypsinized and single cell suspensions were made and equal number of cells were added to the confluent layer of another permissive cell, RK-13 in 6-well plates and were allowed to adhere for 30 min. Cells were overlaid with 0.75% CMC in growth medium and incubated at 37 °C for 96 h. 6-well plates were then washed and stained with 1% crystal violet in 10% buffered formalin. Numbers of plaques were counted and infected cell numbers in total cell suspension were determined. The data represent means with standard errors from 3 independent experiments. rEqIFN-α treatment did not alter the cell numbers infected with T953 when compared with the cells not treated with IFN.
The infectious center assay showed no statistically significant differences in the infected cell numbers between rEqIFN-α treated and untreated EECs (Fig 3.15). Results from 3.13 and 3.15 suggest that exogenous rEqIFN-α treatment of EECs neither blocked viral replication in those cells nor hindered the entry of the virus reducing the infection rate. Rather, the results support the hypothesis that rEqIFN-α pre-treatment prolonged the multi-cycle infection process of the virus resulting in reduced numbers of progeny viruses at the beginning which increased at later time points (48 h.p.i.).

3.13 Effect of T953 Infection on the Type-I IFN Signaling Pathways

Because T953 virus was very resistant to the inhibitory effect of exogenous rEqIFN-α, this raises the question whether the virus was interfering with the type-I IFN mediated JAK-STAT signaling. It is common for different herpesviruses to target JAK-STAT signaling to subvert host interferon responses. To elucidate whether T953 interferes with JAK-STAT signaling, EECs were infected with T953 and, at 30 min before the indicated time points, cells were stimulated with exogenous rEqIFN-α at a concentration of 1000 IU/ml of media. Mock infected EECs treated with rEqIFN-α were used as positive control. At the indicated time points, cells were lysed to prepare whole cell lysate and equal amounts (30 μg) of protein lysates were analyzed by Western blot using anti-pSTAT-1, anti-STAT-1, anti-β-actin and anti-IE1 antibodies. The results showed that T953 virus did not prevent exogenous rEqIFN-α induced STAT-1 phosphorylation at early time points (3 h) but the virus down-regulated STAT-1 phosphorylation at late time points (12 h) (Fig 3.16 A).
Figure 3.16: T953 downregulates STAT-1 phosphorylation. EECs were infected with T953 at MOI of 5 and cells were either left untreated or treated with rEqIFN-α at 1000 IU/ml of media for 30 min before lysis at indicated time points. Equal amounts (30 μg) of whole-cell lysates were separated on 10% bis-cross-linked polyacrylamide gels and subjected to Western blot analysis using (A) pSTAT-1, (B) STAT-1, (C) β-actin, and (E) EHV-1 IE1 antibodies. T953 downregulated the phosphorylation of STAT-1 at later time points but did not alter the level of endogenous STAT-1 level. (D) Densitometric analyses were done to normalize the pSTAT-1 and STAT-1 levels against β-actin by NIH ImageJ software. The Western blot images were representative of 3 different blots from independent experiments.
But this down-regulation of pSTAT-1 at later time points were not due to the down-regulation of endogenous level of STAT-1 since the virus did not alter the endogenous level of STAT-1 (Fig 3.16 B). T953 infection to EECs was confirmed by the expression of EHV-1 IE1 protein (Fig 3.16 E).

3.14 Role of T953 Infection in the Translocation of STAT-1 in EECs
The Western blot findings of T953 mediated interference with JAK-STAT signaling pathways by down-regulating the phosphorylation of STAT-1 molecule was further studied by indirect immunofluorescence assays. EECs were infected with T953 for 12 h, and at 30 min prior to fixation in 4% PFA, infected cells were either treated with rEqIFN-α (1000 IU/ml of media) or with MEM. Mock infected EECs treated with rEqIFN-α were used as positive control. When cells were stained with anti-STAT-1 Ab, the majority of STAT-1 molecules were in the cytoplasm of the mock infected EECs (Fig 3.17.I). But when mock cells were stimulated with IFN the majority of the STAT-1 molecules were translocated into nucleus (Fig 3.17.I). On the other hand, in the absence of rEqIFN-α treatment the majority of the STAT-1 molecules were present in the cytoplasm of T953 infected EECs (Fig 3.17.II). Cytoplasmic STAT-1 in T953 infected EECs indicated that T953 did not activate STAT-1 molecules and thus did not activate JAK-STAT signaling pathways.
Figure 3.17.I: rEqIFN-α induces nuclear translocation of STAT-1. Mock infected EECs were either stimulated with rEqIFN-α at 1000 IU/ml or equal volume of MEM for 30 min prior to fixation in 4% PFA at 12 h.p.i. Cells were then stained with anti-STAT-1, and anti-gC (EHV-1) Abs. A, B, C, and D shows nuclear stain DAPI (blue), STAT-1 (green), EHV-1 gC (red) and merged images respectively. Mock infected EECs in the absence of rEqIFN-α (upper panel) shows cytoplasmic STAT-1 while rEqIFN-α treated mock cells (lower panel) showed nuclear STAT-1.
Figure 3.17.11: T953 infection inhibits nuclear translocation of STAT-1. T953 infected EECs were either stimulated with rEqIFN-α at 1000 IU/ml or equal volume of MEM for 30 min prior to fixation in 4% PFA at 12 h.p.i. Cells were then stained with anti-STAT-1, and anti-gC (EHV-1) Abs. A, B, C, and D shows DAPI nuclear stain (blue), STAT-1 (green), EHV-1 gC (red) and merged images respectively. Both in the absence (upper panel) or presence (lower panel) of rEqIFN-α treatment, the majority of the STAT-1 molecules in the T953 infected EECs were cytoplasmic.
Moreover, T953 infection also prevented rEqlFN-α induced nuclear translocation of STAT-1 (Fig 3.17.II). These findings from the IFA study corroborated the results of the western blot data in the previous section that T953 interfered with JAK-STAT signaling pathways.

3.15 Role of Viral Gene Expression on Type-I IFN Signaling Pathways

It was clear that T953 infection could suppress the activation of STAT-1 molecules and also prevented the nuclear translocation of STAT-1, but it was not evident whether viral gene expression was necessary to exert that effect. Studies were designed to evaluate the role of viral gene expression in JAK-STAT signaling pathways. EECs were infected with WT T953 or UV inactivated T953 (UV T953) and stimulated with rEqlFN-α for 30 min before harvesting cells for total cellular lysates at 18 h.p.i. In the previous results (Fig 3.16) T953 infection for 12 h caused a down-regulation in the STAT-1 phosphorylation but the study was not continued beyond that time points. Protein lysates were analyzed by Western blotting using anti-pSTAT-1, anti-STAT-1 and anti-β-actin Abs. Mock infected cells treated with rEqlFN-α was included as positive controls. Besides showing the role of viral gene expression in suppressing STAT-1 activation, this study also showed whether the suppression effect was continued beyond 12 h.p.i.

The results showed that WT T953 infection suppressed STAT-1 phosphorylation at 18 h.p.i. just as shown above at 12 h.p.i. UV T953, however, did not inhibit rEqlFN-α induced STAT-1 phosphorylation (Fig 3.18). Neither WT T953 nor UV T953 degraded STAT-1 from its endogenous level. These findings suggest that viral gene expression which is absent in UV-T953 infected cells, was necessary to interfere with STAT-1 activation.
Figure 3.18: UV inactivated T953 cannot prevent rEqIFN-α induced STAT-1 phosphorylation. EECs were mock infected or infected with wild type (WT) T953 or UV inactivated T953 or left untreated. Cells were then either induced with exogenous rEqIFN-α at 1000 IU/ml of media or left untreated 30 min before lysis at 18 h.p.i. Equal amounts (30 μg) of whole-cell lysates were loaded into each well of 10% bis-cross-linked polyacrylamide gel and electrophoresed and blotted using (A) anti-pSTAT-1, (B) cocktail of anti-STAT-1 and anti-β-actin antibodies. The Western blot images were representative of 2 different blots from independent experiments.
3.16 Effect of ORF1/2 Deletion on STAT-1 Phosphorylation

ORF1/2 of EHV-1 are the early genes of EHV-1 and have been shown to be involved in host immune responses by modulating cytokine responses such as IFN-γ [342]. ORF1 of the neuropathogenic EHV-1 Ab4 strain has been shown to down-regulate the expression of MHC-I molecules which might interfere with the antigen presentation resulting into the interference of T-cell mediated immune responses [106]. Hence, considering the importance of ORF1 and ORF2, its effect on the activation of JAK-STAT signaling pathways was evaluated using ORF1 and ORF2 deletion mutants of neuropathogenic strain Ab4 (Δ1/2 Ab4). EECs were infected with WT Ab4 virus, or Δ1/2 Ab4 mutant virus at low MOI (MOI=1) for 18 h. Before lysis cells were induced with rEqIFN-α for 30 min. Western blot analyses showed that Δ1/2 Ab4 virus infected EECs could not prevent rEqIFN-α induced phosphorylation of STAT-1 when compared with rEqIFN-α stimulated mock infected EECs (Fig 3.19 A). On the other hand, WT Ab4 infected EECs suppressed STAT-1 phosphorylation when stimulated with exogenous rEqIFN-α (Fig 3.19 A). Neither WT Ab4 nor Δ1/2 Ab4 infection down-regulated the endogenous level of STAT-1 (Fig 3.19 B). The membranes were also blotted with EHV-1 IE1 Ab to check that WT as well as mutants of Ab4 viruses successfully infected EECs (Fig 3.19 E). The results indicate that EHV-1 genes encoded by ORF1 and ORF2 suppressed exogenous rEqIFN-α induced STAT-1 phosphorylation.
Figure 3.19: Δ1/2 Ab4 mutant virus cannot prevent STAT-1 phosphorylation. EECs were infected with another EHV-1 neuropathogenic strain Ab4 WT, or with an ORF1/2 deletion mutants of Ab4 (Δ1/2 Ab4) at MOI of 1 and cells were either left untreated or treated with rEqIFN-α at 1000 IU/ml of media for 30 min before lysis at 18 h.p.i. Equal amounts (30 μg) of protein lysates were separated on 10% bis-cross-linked polyacrylamide gels and subjected to Western blot analysis using (A) anti-pSTAT-1, (B) anti-STAT-1, (C, F) anti-β-actin and (E) anti-EHV-1 IE1 antibodies. (D) Densitometric analyses were done to normalize the pSTAT-1 and STAT-1 levels against β-actin by NIH ImageJ software. The Western blot images were representative of 3 different blots from independent experiments.
3.17 Deletion of ORFs 1 and 2 Does Not Interfere with the Entry and Gene Expression of Mutant Δ1/2 Ab4 virus

The mutant virus Δ1/2 Ab4 was characterized by Soboll Hussey et al. [342] earlier in detail and a growth curve was made on NBL-6 cells. It was shown that deletion of ORF 1 and 2 does not lower the infectivity of the mutant virus when compared to its WT Ab4 virus. However, in this study also it was evaluated whether the Δ1/2 Ab4 mutants were able to infect and express its genes in the EECs by IFA study using antibodies against EHV-1 IE1 protein and also EHV-1 late protein gC. The IFA data showing the expression of IE1 protein indicated that Δ1/2 Ab4 mutant was able to enter into EECs successfully like its parental virus Ab4 (Fig 3.20). The expression of late protein gC also confirmed that the mutant virus was able to infect and express its genes in EECs like WT Ab4 (Fig 3.20).
Figure 3.20: ORF1/2 deletion does not affect the capacity of Δ1/2 Ab4 mutant virus to infect EECs. Panels show images of i) mock infected EECs, ii) Ab4 WT infected EECs and iii) Δ1/2 Ab4 infected EECs. A, B, C and D represents DAPI (nuclear stain) stained images, anti-IE1 Ab (green) stained images, anti-gC Ab (Red) stained images and (D) Merged images of A, B, and C respectively.
3.18 Effect of T953 Infection on the Expression of Viperin in EECs

Type-I IFNs exert their antiviral effects through the induction of ISGs. As EHV-1 T953 interfered with the activation of STAT-1 molecules, which is important for downstream ISG induction, it was imperative to know if the virus was able to inhibit ISG induction partially or completely. Studies were designed to find out if T953 infection of EECs induced expression of a recently discovered IFN induced antiviral protein, viperin. As reviewed earlier that viperin has been found to inhibit many DNA as well as RNA viruses such as HCMV, HCV, dengue virus, influenza virus and many others. EECs were infected with T953, or treated as indicated (Fig 3.21). At 3 and 12 h.p.i whole cell lysates were prepared and equal amounts of protein lysates were subjected to Western blot analysis using anti-viperin and anti-β-actin Abs. Results indicate that T953 infection did not induce viperin protein at either early (3 h) or later (12 h) time points (Fig 3.21. Interestingly, T953 infection at later time points also suppressed poly I:C induced viperin expression. The results also indicated that T953 infection for 3 h did not prevent poly I:C induced viperin expression. The Western blot image also showed that late gene blocking of T953 by PAA treatment permitted viperin protein expression compared to T953 infected EECs without PAA. In summary, T953 virus infection of EECs suppressed viperin expression and the virus also had the ability to inhibit other stimuli that induce viperin expression.
Figure 3.21: T953 infection suppresses viperin expression. EECs were infected with T953, or treated with poly I:C, or coinfected as indicated. At indicated time points whole cell lysates were prepared and equal amounts (30 μg) of proteins were separated on 10% SDS-PAGE and analyzed by Western blotting using anti-viperin and anti-β-actin Abs. (A) At 3 h.p.i. T953 neither induced viperin expression nor completely prevented poly I:C induced viperin expression. (B) At 12 h.p.i. T953 prevented poly I:C induced viperin expression.
3.19 T953 Infection Interferes with Exogenous IFN-α induced ISGs Viperin and ISG56 Expression

Because T953 infection did not induce any viperin protein either at early and later time points and viperin could be induced by both IFN-dependent as well as IFN-independent pathways, further studies were designed to determine whether the virus was able to inhibit exogenous rEqIFN-α-induced viperin expression both at mRNA and protein level. In addition to viperin, ISG56 was also evaluated in this study.

EECs were infected with T953 and at 6 h.p.i. stimulated with rEqIFN-α for another 1 or 6 h. Mock infected EECs were also stimulated with rEqIFN-α for 1 and 6 h. At the indicated time points transcription of viperin and ISG56 mRNA were evaluated by real-time RT-PCR assay. Results indicate that T953 infection of EECs for 6 h reduced transcription of rEqIFN-α-induced viperin mRNA significantly (Fig 3.22 A). The virus infection of EECs for 6 h also down-regulated rEqIFN-α-induced ISG56 mRNA significantly (Fig 3.22 B).
Figure 3.22: T953 inhibits interferon induction of ISGs at the transcriptional level. EECs were either mock infected or infected with T953 at an MOI of 5. At 6 h.p.i., infected EECs were stimulated with exogenous rEqIFN-α (1000 IU/ml of media) for either 1 h or 6 h. One group of mock as well as virus infected cells was left untreated. At indicated time points, cells were lysed for total cellular RNA and mRNA transcripts of ISGs were quantitated by real-time RT-PCR assays. T953 inhibited exogenous rEqIFN-α induced viperin (A) as well as ISG56 (B) mRNA transcription significantly (***= p<0.001 and *= p<0.05). Data represent means with standard errors from 3 independent experiments.

The suppression of viperin and ISG56 transcription was confirmed by Western blot analyses of the T953 infected cell lysates (Fig 3.23).
Figure 3.23: T953 inhibits interferon induced ISGs induction at translational level. EECs were either mock infected or infected with T953 for 6 h. Cells were then either stimulated with exogenous EqIFN-α (1000 IU/ml of media) or left unstimulated for another 6 h. Whole-cell lysates were prepared and equal amounts of lysates were subjected to Western blot analysis by using (A) anti-viperin, (B) anti-ISG56 and (C) anti-β-actin Abs. T953 downregulated protein level of viperin and also reduced ISG56 protein translation induced by EqIFN-α. Images are representative of 3 independent experiments.

EECs were infected with T953 for 6 h and stimulated with rEqIFN-α for another 6 h. Whole cell lysates were prepared and equal amounts (30 μg protein) of lysates were subjected to Western blot analyses using anti-viperin, anti-ISG56 and anti-β-actin Abs. The results showed that T953 infection down-regulated rEqIFN-α induced viperin protein expression by more than half (3.23 A). On the other hand, T953 infection to EECs suppressed rEqIFN-α induced ISG56 completely (3.23 B). Altogether, these data indicate that T953 infection suppressed rEqIFN-α induced ISGs such as viperin and ISG56 both at the transcriptional as well as translational levels.

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CHAPTER IV: DISCUSSION

The first line of defense against infectious agents is mounted by the host’s innate immune system which was thought to be very simple [352] as until recently it was presumed that the adaptive immune system played the central role in the clearance of viral infections. It is now believed that innate immune system plays the crucial roles in acute infections especially in viral infections [353]. Generally, in microbial infections, innate immune system exerts its action by recognizing pathogens through different innate immune receptors (TLRs, RLRs and NLRs) and then secreting different types of cytokines to eliminate that infection from the host. Failing to do so, innate immune system recruits the adaptive immune system to eventually clear the infection [352]. It is now well documented that the type-I IFNs, namely IFN-α and IFN-β act as nonspecific antiviral agents against many viruses, including herpesviruses [314]. Besides inhibiting virus replication directly, type-I IFNs have also been reported to modulate adaptive immune responses by enhancing CD8+ T cell activation, regulating migration of immune cells, helping in the maturation of DC and also inducing apoptosis [300, 354, 355].

4.1 Suppression of Type-I IFN by EHV-1 T953 Strain After an Initial Transient Induction

Because effective replication of EHV-1 in endothelial cells is thought to be necessary for the development of EHM as well as also for abortions, control of virus replication in endothelial cells could be an effective way to control EHV-1 abortions and EHM. The central hypothesis of this research was that EHV-1 has mechanisms to evade the antiviral effects of type-I IFN response of the horse. The suppression of type-I IFN responses at either IFN induction or IFN signaling or both could make the microenvironment conducive for efficient lytic replication of EHV-1 leading to the inflammatory damage to the blood vessel and surrounding tissues. To date, there have been no detailed reports of type-I IFN responses to EHV-1 infection in endothelial
there have been no detailed reports of type-I IFN responses to EHV-1 infection in endothelial cells. Herpes viruses like HSV-1, BHV-1, VZV have been shown to inhibit type-I IFN production in fibroblast cells [303, 305, 356]. In contrast to this, HSV-1 has also been shown to induce the production of type-I IFN mainly by pDC indicating that induction of interferon responses is cell type specific [357]. In this study the type-I IFN responses to EHV-1 infection were investigated in EECs, as a model for simulating the infection in horses.

EHV-1 infection of EECs caused a transient induction of IFN-β mRNA at an early stage of infection (3 h.p.i.) but soon after that the virus started suppressing further induction of IFN-β mRNA and by 12 h.p.i., the virus shut off the transcription of IFN-β gene to a basal level when compared to mock infected cells. However, when measured at protein level by type-I IFN bioassay, it was observed that the virus infection showed type-I IFN activity in infected cell supernatants both at 3 h and at 12 h although reduced activity at later time points (12 h). The type-I IFN activity at protein level found in the later time points could be due to the accumulating effect of type-I IFNs that were secreted in 3 and 6 h.p.i. In contrast to this study, Edington et al. [332] observed that in vivo EHV-1 infection of horses induced high level of EqIFN in serum. Also, low levels of EqIFN were secreted by explanted mononuclear cells infected with EHV-1. In another in vitro study, Wagner et al. [349] found that EHV-1 infected PBMCs induced type-I IFN (IFN-α) and chemokines like CCL5, CXCL9 and CXCL10 but suppressed CCL2 and CCL3 production. Although, the data from this study appears contradictory to the previous studies, upon careful evaluation it should be clear that the current results showed that EHV-1 was capable of inducing type-I IFN (IFN-β) gene expression (both at mRNA and protein level) in the endothelial cells. Furthermore, the kinetic analyses of type-I IFN production by EHV-1 infected EECs indicated that the virus also had the ability to interfere with later type-I IFN production, i.e., after an initial phase of induction. On the other hand, the previous studies, either in vivo (horse) or in vitro (equine PBMCs) did not address whether EHV-1 could also inhibit IFN-α/β production. The
apparent differences in EHV-1 induced type-I IFNs could be attributed to a number of factors. In
the case of in vivo infection, a number of different cell types both immune cells (pDC, mDC and
PBMCs) as well as non-immune cells (epithelial, endothelial and fibroblast cells) are present in
the body and which specific cell type(s) produced the IFN response in the serum of EHV-1
infected horses was not specified. Research has shown that type-I IFN induction is cell type
specific and immune cells like DCs could respond differently than non-immune cells to a virus
infection [358, 359]. Indeed, Le et al. [360] and others [359] reported that mouse
cytomegalovirus (MCMV) infected pDC produced a significant amount of type-I IFNs, but non-
immune cells like fibroblasts did not. By inhibiting type-I IFN production in fibroblasts, MCMV
made an ‘intracellular milieu’ or ‘virus factory’ which was optimized for viral genome replication
[360]. pDCs have been reported to be the cells that primarily produce type-I IFNs in response to
infectious agents [361]. It is entirely possible that during in vivo EHV-1 infection, pDCs along
with other cells like mDCs and other mononuclear cells are activated to produce IFN-α/β which
was detected by Edington’s group. Wagner’s group also found IFN-α in EHV-1 infected PBMCs
(immune cells). Difference due to virus strains could not be ruled out since Wagner’s group used
different strains of EHV-1 (Ab4, NY03 and RacL11) than the strain used in this study (T953).
The relation of type-I IFN production to the strain differences needs to be further investigated.

4.2 T953 Virus Mediated Shut-down of Type-I IFN Induction by SeV or Poly I:C

Next, it was important to determine if the virus actively shut down type-I IFN production in
the host cells or the suppression of IFN-α/β at later time points was due to normal IFN production
kinetics. To address that issue, a co-infection study was designed where EECs were infected
either with T953 and SeV together or T953 and poly I:C together for different periods of time. In
both cases results showed that T953 suppressed the SeV or poly I:C induced type-I IFN by EECs
(Fig 3.3 and 3.4).
Interestingly, when poly I:C treated EECs were taken as positive control, a biphasic induction of IFN-β gene transcription was found. After a peak of IFN-β mRNA at 3 h there was a downregulation of IFN-β mRNA at 6 h and again at 12 h there was increase in the IFN-β gene transcription (Fig 3.3). This phenomenon was also seen at the translation level (Fig 3.4). This biphasic induction of IFN-β was probably due to a positive feedback loop. Using an IFN-β-GFP reporter system, Hwang et al. [362] showed that synthetic dsRNA analogue, poly I:C causes a biphasic expression of IFN-β in the HepG2 cell line in a positive feedback loop. Poly I:C also has been found to induce STAT-1 phosphorylation in a biphasic manner [363]. Type-I IFNs have been shown to induce the expression of a number of ISGs, some of which like RIG-I and MDA-5 are also involved in sensing foreign dsRNA [362, 364-366]. For example, secreted IFN-β stimulates the RLRs like RIG-I and MDA5 which stimulates autocrine feedback loop producing more IFN-β. IFN-β induction through RLRs involves a number of positive feedback loops which also involve multiple ISGs. IFN-β also stimulates LGP2 which competes with RLRs for binding of foreign cytosolic RNA to activate MAVS/IPS-1 in mitochondria to produce more type-I IFNs in a positive feedback manner [365]. The transcription factor IRF-7 which is an ISG plays an important role in the production of type-I IFNs [367]. It has been reported earlier that IFN-β induces the production of IRF-7 which in turn transactivates the transcription of more type-I IFNs resulting in a positive feedback loop [367]. Another reason for the biphasic expression of IFN-β could be the stochastic chromatin states before initiation of transcription, resulting in differential gene expression as has been found in the bimodal expression of IL-4 [362]. It has been shown that the biphasic expression of IL-4 due to the heterogeneous state of the chromatin in Th cells was not controlled by a positive feedback loop [362, 368]. However, chromatin state has not been found to be the principal reason for biphasic IFN-β gene expression since, blocking of IFN-β secretion disrupted this pattern indicating a positive feedback loop by secreted IFN-β [362]. If this bimodality was due to the heterogeneous status of the chromatin at IFN-β gene locus, blocking of IFN-β-loop would not have any effect. In the present study biphasic IFN-β gene
expression induced by poly I:C probably involved a positive feedback loop which caused a second peak at 12 h.

To verify that EHV-1 gene expression actively shut down the type-IFN production, another co-infection study was designed. In the earlier co-infection studies (Fig 3.3, 3.4) combinations of viruses (T953 and SeV) or combinations of virus and chemical (T953 and poly I:C) were added to cells at the same time and incubated for same duration. But in this co-infection experiment cells were pre-infected with T953 and the virus was allowed to express its genes for some time (6h) before the cells were coinfected with SeV to induce IFN-β induction and the results indicated that EHV-1 gene expression actively suppressed the ability of SeV for IFN-β mRNA induction (Fig 3.5). Sometimes, when a virus infected cells are co-infected with another different virus, entry of the second virus may be interfered. It could be argued that inhibition of SeV induced IFN-β gene transcription was due to the interference of SeV entry by T953. To test this another co-infection was done where cells were infected with T953 for different time periods (0 h, 3 h, 6 h and 12 h) and then induced with poly I:C for IFN-β gene expression (Fig 3.6). The inhibition of both SeV and poly I:C (Fig 3.5, 3.6) induced IFN-β gene transcription by T953 virus confirmed that the T953 virus not only suppressed IFN induction from its own stimulus but actively shut down type-I IFN induction induced by other exogenous stimuli.

It is noteworthy that UV inactivated T953 virus did not induce any significant type-I IFNs (Fig 3.3, 3.4) at all-time points suggesting that active viral gene expression is necessary for the initial IFN-α/β production. It was expected that PRRs would recognize the UV inactivated T953 and produce type-I IFNs which did not happen in this study. It is possible that UV inactivated T953 virus did not have the threshold level stimuli to initiate the type-I IFN production.
4.3 Relationship of EHV-1 Late Gene(s) to Suppression of Type-I IFN Induction

There was an indication that EHV-1 late genes might be responsible for the inhibition of type-I IFN induction as at later time points post infection there was no significant IFN production. Since the expression of EHV-1 late genes is dependent on the replication of viral DNA by DNA polymerase, the expression of EHV-1 late genes were blocked by inhibiting EHV-1 DNA polymerase using PAA. Although there are several reports that PAA preferentially inhibits herpesviral DNA polymerase and thereby inhibits the late gene expression of herpesvirus [369] we verified that PAA also blocked EHV-1 DNA polymerase without host cell toxicity. The immunofluorescence data (Fig 3.7) showed that in the presence of PAA, the T953 virus failed to express a marker late gene (gC) while expression of a marker IE gene (IR1) was unaffected.

Interestingly, when EHV-1 late gene expression was blocked, a robust type-I IFN response both at mRNA as well as protein level (Fig 3.8 A and 3.8 B) was observed at later time points (12 h and 18 h). At these time points IFN was undetectable in the T953 infected EECs if late gene expression was not blocked. This result indicates that viral late gene(s) are partly or completely responsible for the anti-IFN activity. However, the specific gene(s) responsible for this inhibitory effect have not been determined and further investigations are required.

4.4 Mechanism(s) of Suppression of Type-I IFN Induction by T953 Virus

To explore the mechanism(s) EHV-1 uses to suppress IFN production at later time points, additional studies were carried out to determine whether EHV-1 interferes with any of the steps in the activation of the principal pathways for type-I IFN production, such as IRF-3 signaling pathways. A number of different viruses from diverse families have been reported to interfere with IRF-3 signaling pathways [303, 307, 331, 360, 370-372]. From the recognition of PAMPs by PRRs to the production of IFN-β through IRF-3 signaling pathways, multiple signaling molecules and multiple steps are involved and viruses, therefore, have multiple opportunities to evade this important host response. While some viruses degrade the endogenous level of IRF-3, others
inhibit its activation by preventing phosphorylation, or dimerization, or even nuclear accumulation of activated IRF-3 [303, 306, 371, 373]. While other viruses that cannot interfere in the activation of IRF-3, they may interfere with the interaction between CBP/p300 and IRF-3 [306]. Alphaherpesvirus HSV-1 was shown to suppress IRF-3 hyperphosphorylation as well as IRF-3 mediated ISG promoter activity by HSV-1 IE protein ICP0 [307]. HSV-1 however has not been reported to degrade endogenous IRF-3 protein [307]. On the other hand, BHV-1, another alphaherpesvirus, but a different genus, Varicellovirus through its IE protein ICP0 degrades endogenous level of IRF-3 protein to inhibit type-I IFN production [303]. Therefore, it was thought that EHV-1 being a Varicellovirus could adapt the same technique in order to evade host type-I IFN response. The Western blot (WB) data (Fig 3.9) indeed indicated that T953 virus down-regulates endogenous level of IRF-3 protein at later time points (6 h.p.i. and 12 h.p.i.) when compared to mock infected cell lysates. It is also to be noted that the down-regulation of IRF-3 protein increased with the increase of the amount of late protein gC expression. This suggested that expression of T953 late gene(s) could be responsible for the degradation of IRF-3 at later time points resulting into the suppression of IFN production. Another possibility is that the down-regulation of the total amount of endogenous IRF-3 protein at later time points could be nonspecific and it is due to the global shut down of gene expression after EHV-1 infection. This seems less likely to happen, since unlike HSV-1, EHV-1 infection does not show much inhibition of global gene expression [108].

Many viruses evolve multiple targets to interfere with IRF-3 mediated signaling pathways. HSV-1 inhibits nuclear accumulation of IRF-3, induces degradation of activated IRF-3, and interferes with the binding of IRF-3 to CBP/p300 required transcriptional activation of different genes [306]. Therefore, it was also interesting to explore whether T953 evolved similar multiple strategies to subvert IRF-3 signaling pathways. Data from this study (Fig 3.10) show that at an early time points (3 h) T953 infection of EECs caused IRF-3 phosphorylation which was reduced at a later time points (12 h). The WB data from the subcellular fractionized cell lysates also
showed that initially the majority of the pIRF-3 molecules were located in the nucleus while cytoplasmic pIRF-3 increased at later time points. This phenomenon suggests that at the beginning of the infection IRF-3 is activated and translocated into the nucleus from the cytoplasm and this activated pIRF-3 might stimulate downstream signaling resulting in the IFN-β transcription that was observed at earlier time points in this study. At later time points the observed downregulation of pIRF-3 molecules in the nucleus explained why IFN-β was not found during that time. But a specific reason for this downregulation of pIRF-3 molecules in the nucleus was not identified in this study. However, T953 virus-induced down-regulation of endogenous IRF-3 molecules would likely be the major reason. In addition to this, it is possible that some unidentified viral proteins (late gene products) might interact with pIRF-3 and trigger the translocation of pIRF-3 back to the cytoplasm from the nucleus, downregulating the level of pIRF-3 in the nucleus. A similar mechanism has been proposed by Melroe et al [306] for HSV-1: viral ICP0 targets activated IRF-3 molecules for posttranslational modification to translocate back to the cytoplasm. An alternative possibility is that an unidentified T953 viral protein(s) could interact with activated IRF-3 (pIRF-3) and trigger its degradation at later time points. It has been shown that rotavirus NSP1 protein interacts with activated IRF-3 and enhances its degradation through cellular proteasomes [374]. It has been suggested that ICP0 of HSV-1 also associates with IRF-3 and targets activated IRF-3 molecules through the E3-ubiquitin ligase for enhanced ubiquitination and degradation [306].

When cell lysates were analyzed with IRF-3 Ab rather than pIRF-3 Ab, it was observed that IRF-3 was translocated into the nucleus suggesting activation of IRF-3 at the early time points when IFN-β was also expressed. But at later time points the level of nuclear IRF-3 was drastically downregulated. Densitometric analyses of the band intensity also indicated that the total cytoplasmic IRF-3 level was increased at 12 h.p.i. compared to 3 h.p.i. This indicated that either IRF-3 phosphorylation was downregulated reducing the total nuclear level of IRF-3, or translocation of pIRF-3 to the nucleus from the cytoplasm was inhibited at later time points. In
either case there would be reduced nuclear IRF-3 but comparatively increased cytoplasmic IRF-3. But when the ratio of pIRF-3/IRF-3 was calculated to find out the fraction of IRF-3 molecules that was activated, it was observed that although the ratio was decreased in the nucleus from 3 h to 12 h, there was also an increase in the ratio in the cytoplasm, and the total pIRF-3/IRF-3 ratio (cytoplasmic + nuclear) was not altered much. This indicated that perhaps T953 infection prevented pIRF-3 nuclear translocation from the cytoplasm at later time points rather than preventing phosphorylation of IRF-3.

The IFA data (3.11) also suggested that T953 infection induces IRF-3 activation followed by nuclear translocation as early as 1 h.p.i. and peaks at 3 h.p.i. At 6 h.p.i., although the majority of T953 infected cells showed nuclear IRF-3, a number of cells also showed cytoplasmic IRF-3 indicating T953 infection either reduced IRF-3 activation or reduced translocation into the nucleus. At 12 h.p.i. however, the majority of cells showed cytoplasmic IRF-3 suggesting the virus infection prevents nuclear accumulation of IRF-3. Therefore, whether T953 infection degrades activated IRF-3 or prevents activation of IRF-3 or inhibits translocation of activated IRF-3 is not clear in this study and further studies are required to specifically identify the step(s) in the IRF-3 signaling pathways at which virus interfered. But it is certainly clear that in all cases T953 infection induced down-regulation of endogenous IRF-3 and also prevented nuclear accumulation of IRF-3 at later time points thus down-regulating IRF-3 signaling pathways. These interferences in the IRF-3 signaling pathways probably were responsible for the absence of IFN-β in the later time points.

This study was also intended to see whether T953 virus infection also inhibited NF-κB signaling pathways as the activation of the transcription factor NF-κB also plays a role in IFN-β induction [375]. In the inactive state, the NF-κB subunits, p50 and p65 are associated with an inhibitory protein IκBα and thus sequestered in the cytoplasm [351, 376]. When activated by different stimuli like LPS or infectious agents like virus or bacteria, the inhibitory protein IκBα is phosphorylated by cellular kinases and then degraded by cellular proteasomes resulting in the
activation of p50 and p65 subunits which then translocate into the nucleus to transactivate transcription of a diverse set of genes [376]. In this study it was observed that T953 virus infection induced degradation of the inhibitory protein IκBα (Fig 3.11.i) at later time points (12 h.p.i.) indirectly suggesting an activation of the NF-κB signaling pathways. During the early phase of the infection IκBα was not degraded indicating that at early time points (3 h.p.i.) NF-κB signaling pathways were not activated. The IFA data (Fig 3.12.ii) using anti-p50 Ab showed that at early time points the p50 protein was located in the cytoplasm of T953 infected cells. But at the later time points p50 protein was found to be translocated from the cytoplasm into the nucleus in T953 infected cells. This indicates that T953 infection of EECs does not activate NF-κB signaling pathways at early times post infection but at later times post infection the virus activates the transcription factor NF-κB. This also indicates that probably the type-I response induced by T953 infection at early time points is not due to the involvement of NF-κB signaling pathways, rather, it is a result of the activation of IRF-3 signaling pathways. Also at later time points, the suppression of type-I IFN is probably not due to the inhibition NF-κB signaling pathways but due to interference with IRF-3 signaling pathways at multiple levels. By activating NF-κB signaling pathways at later time points, the virus may trigger the expression of NF-κB dependent gene expressions especially expression of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α. These proinflammatory cytokines in turn could play vital role in the inflammation of the blood vessels of uterus and CNS causing EHV-1-induced pathology [377]. Again by activating the expression of NF-κB dependent genes, the virus also could trigger cell survival [377] which in turn would be advantageous to the virus for its replication. Some viral proteins like African Swine Fever virus protein A238L interact with p300 protein and inhibit the association between NF-κB-p65 and p300 which is required for transactivation of genes and thus prevents NF-κB signaling mediated gene transcription [378]. Adenovirus protein E1, Simian Virus 40 (SV40) T large Ag and E6 or E7 protein of human papillomavirus (HPV) have also been reported to interact with p300 and inhibit the association of NF-κB subunit and CBP/p300 [379-382]. As a result of this, in
spite of activation of NF-κB signaling pathways sometimes there is no transcription of NF-κB dependent genes. Therefore, an alternative explanation could be that in addition to IRF-3 activation pathways, NF-κB signaling pathways are also inhibited but downstream to the activation and nuclear accumulation of the transcription factor NF-κB. It is entirely possible that T953 virus proteins may interact with CBP/p300 and interfere with the association between activated NF-κB subunits and CBP/p300 which also could suppress type-I IFN production at later time points.

Taken together, these data indicate that during the early stage of the T953 infection in vitro in EECs, the host cells respond by inducing a type-I IFN response. At the late stage of the infection, however, viral late proteins interfere with the IRF-3 signaling pathways by inducing down-regulation of IRF-3 as well as by interfering with the translocation of activated IRF-3 into the nucleus from the cytoplasm. Further studies are required to confirm whether the NF-κB signaling pathways are also inhibited at single or multiple steps by T953 virus infection. This study was not focused on the identification of the specific proteins of T953 virus that may have a role in suppressing type-I IFN production by interfering with IRF-3 signaling pathways. Future studies in this area are very important in order to identify the specific viral proteins that could help understand the virus-host interaction better and may provide insights in designing of future therapies against this viral disease.

4.5 Resistance of T953 Virus to the Effect of rEqIFN-α

The next part of this study was focused on characterization of the efficacy of type-I IFNs against T953 infection. As reviewed earlier, type-I IFNs bind to its cognate receptors IFNAR1 and IFNAR2 which trigger a cascade of signaling events (JAK-STAT signaling) resulting in the activation of STAT-1and STAT-2 proteins by phosphorylation [278]. Phosphorylated STATs then dimerize and enter into the nucleus and bind to the interferon stimulated response elements (ISRE) of a number of ISGs enhancing their transcription [288]. These ISGs actually inhibit replication of viruses from diverse families through various mechanisms [288]. It was very
important to know whether T953 was sensitive or resistant to the inhibitory effects of type-I IFNs. Viruses like SeV and VSV have been shown to be very sensitive to the inhibitory effect of type-I IFNs [383-385]. On the other hand herpesviruses like HSV-1 have been known to be resistant to the effect of IFN-α/β because of the ICP0 protein [386]. In this study also the effect of exogenous rEqIFN-α on the replication of EHV-1 T953 strain in vitro in EECs was evaluated. It was observed that pre-treatment of EECs with rEqIFN-α at a concentration of 1000 IU/ml for 24 h had very little inhibitory effect on T953 replication indicating that T953 virus is very resistant to the inhibitory effect of IFN-α (Fig 3.13). The IFN-α treatment could not even decrease the number of plaques by 10 fold (1 log scale). On the other hand, replication of IFN-sensitive virus VSV was shown to be completely blocked. Interestingly, the minimal inhibitory effect that was shown by rEqIFN-α against T953 virus at 12 and 24 h.p.i. were reduced at 36 and 48 h.p.i. This could occur if rEqIFN-α treatment prolonged the replication cycle of T953 virus but not completely blocking it. It is also possible that pre-treatment of rEqIFN-α caused interference with the entry of the T953 virus and less number of the virus got entry to the cells. This could explain the lower number of plaques in the beginning of infection (12 and 24 h.p.i.) in IFN-treated cells. But while few viruses entered into EECs and replicated, those progeny viruses then would have infected nearby cells producing more progeny viruses and this increased the titer of the virus at 36 and 48 h.p.i. To investigate that possibility, an infectious center assay was performed and the result showed no differences in the number of infectious centers in IFN-treated and non-treated EECs. This suggests that rEqIFN-α pre-treatment could not restrict T953 replication in EECs but it delayed the replication cycle. By resisting the effect of type-I IFN, the virus may interfere with the MHC-I expression or DC maturation both of which interferes with the Ag presentation to the CTLs. This would be advantageous to the virus for its replication. Although rEqIFN-α pre-treatment did not block EHV-1 replication, other IFNs such as IFN-β have not been tried in this study. However, it is very unlikely that IFN-β could make a significant difference since both IFN-α and IFN-β signal through the same receptors and exert their biological effects through very
similar mechanisms mediated by ISGs. On the other hand, it would be interesting to see if IFN-α, IFN-λ and IFN-γ together had any synergistic effect and whether these combination therapies could restrict EHV-1 replication. There has been multiple reports that replication of HSV is restricted significantly by the synergistic effects of either IFN-α and IFN-γ [387] or IFN-β and IFN-γ [388] combination therapies in fibroblasts as well as in epidermal cells or even in vivo in mice [389]. Therefore, it is possible that same combination therapy may work for EHV-1 also.

4.6 T953 Mediated Suppression of JAK-STAT Signaling Pathways

Because rEqIFN-α had no significant inhibitory effect on T953 replication, it was thought that the virus must be interfering with the effector arm of type-I IFNs, i.e., signaling of type-I IFNs. An alternative explanation could be that the virus does not interfere with JAK-STAT signaling but interferes with the transcription of ISGs which actually provide antiviral immunity to the host cells. It is, however, also possible that T953 virus may not even inhibit ISG induction but could inhibit ISGs by posttranslational modifications such as ubiquitination for enhanced degradation by cellular proteasomes.

To understand the mechanism of resistance against rEqIFN-α inhibitory effect, studies were designed to evaluate whether the virus interfered with JAK-STAT signaling pathways. In the WB results (Fig 3.16) it was observed that in early phase of the infection T953 virus not only activated STAT-1 molecules by phosphorylation but also the virus did not prevent exogenous rEqIFN-α induced STAT-1 phosphorylation. This suggested that T953 virus infection activated JAK-STAT signaling at early time points of infection. However, by late phase of the infection T953 virus inhibited STAT-1 phosphorylation without exogenous rEqIFN-α treatment and the virus also down-regulated exogenous rEqIFN-α induced STAT-1 phosphorylation, indicating an interference with the JAK-STAT signaling.

These findings from WB were confirmed by the IFA (Fig 3.17) showing that T953 down-regulated nuclear accumulation of STAT-1 molecules both in the presence or absence of rEqIFN-
α at later time points of the infection, as the majority of the STAT-1 molecules were in the cytoplasm of the infected cells. The virus infection did not change the endogenous level of STAT-1 molecules based on the WB data (Fig 3.16 B). Therefore, the down-regulation of the pSTAT-1 in T953 infected cells were not due to the down-regulation of the endogenous level of STAT-1. Moreover, the densitometric analyses of the WB image showed that there was a down-regulation in the pSTAT-1/STAT-1 ratio indicating a suppression of STAT-1 phosphorylation in the later time points of the infection. The presence of a majority of STAT-1 molecules in the cytoplasm of T953 infected cells at 12 h.p.i. (Fig 3.17.iii and 3.17.iv) also suggests that in the late phase of infection the virus prevents STAT-1 phosphorylation. It is also possible that T953 infection triggers STAT-1 molecules to translocate back into the cytoplasm from the nucleus at 12 h.p.i. In both cases, however, there is down-regulation of STAT-1 phosphorylation and therefore, the interference with JAK-STAT signaling provides T953 virus with resistance to the inhibitory effect of rEqIFN-α. Similar cases of JAK-STAT signaling inhibition by herpesviruses such as HSV-1, VZV and cytomegalovirus have been reported [313, 390, 391]. HSV-1 ICP27 was shown to be the major protein that inhibits STAT-1 phosphorylation induced by exogenous IFN-α/β [313]. Being an alphaherpesvirus, EHV-1 also might evolve the same strategies to subvert type-I IFN signaling and gain resistance to the inhibitory effects of rEqIFN-α.

The next goal was to determine the importance of viral gene expression for inhibition of JAK-STAT signaling induced by rEqIFN-α. When T953 was UV inactivated to restrict its gene expression, it was observed that inactivated T953 infection could not prevent STAT-1 phosphorylation anymore induced by exogenous rEqIFN-α (Fig 3.18). This indicates that viral gene expression is necessary for the resistance to the inhibitory effect of rEqIFN-α.
4.7 Involvement of ORF1/2 in the Inhibition of JAK-STAT Signaling Pathways

In the next step, studies were designed to identify the viral proteins that were involved in the suppression of type-I IFN signaling. Reports have earlier been published about the importance of EHV-1 ORF1 and ORF2 as virulence factors in the in vivo challenge of horses as well as also in vitro infection [106, 342]. A deletion mutant of Ab4 virus devoid of ORF1 and ORF2 (Δ1/2 Ab4) has been shown to cause decreased interleukin-8 (IL-8) and increased T-bet mRNA expression. Again IFN-β has been reported to decrease IL-8 expression in several reports [392, 393] and T-bet is a transcription factor known to induce the hallmark Th1 cytokine, IFN-γ expression [394]. Therefore, the in vivo study with Δ1/2 Ab4 recombinant virus suggests that the mutant virus likely modulate the IFN-β as well as IFN-γ mediated immune response when compared to WT Ab4 although that study did not involve IFN-β responses. Another study with ORF1 deletion Ab4 (d1 Ab4) mutant virus showed that ORF1 is involved in the EHV-1 induced down-regulation of MHC-I molecules. MHC-I is involved in the Ag presentation by APCs such as DCs and macrophages but MHC-I is also an ISG inducible with type-I IFNs through JAK-STAT signaling [395]. Therefore, these studies raise the possibility that ORF1/2 of EHV-1 could be candidate genes involved in suppressing the STAT-1 phosphorylation at the later phase of the infection. The WB results (Fig 3.19) indeed showed that Δ1/2 Ab4 mutant virus did not prevent STAT-1 phosphorylation induced by exogenous treatment of EECs with rEqIFN-α when compared to its WT parent virus (WT Ab4). There was no difference in the endogenous levels of STAT-1 proteins among mock, Δ1/2 Ab4 and WT Ab4 infected EECs. It could be argued that deletion of ORF1 and ORF2 cause the mutant virus to be inefficient in the entry or replication processes in infected cells. But Dr. Klaus Osterrieder who provided the virus already characterized the virus in RK-13 cells and found that the mutant virus is capable of replicating with titers comparable to WT Ab4 [342]. In this study, however, the entry and gene expression capacity of the Δ1/2 Ab4 mutant virus were analyzed by IFA using anti-IE1 (IE gene product) and anti-gC (L gene product) Abs. The IFA (Fig 3.20) results showed that the mutant virus is capable of entry and
expression of viral genes, indirectly supporting the replication capacity of mutant Ab4 virus. Thus, these findings suggest that EHV-1 ORF1 or ORF2, or ORF1 and ORF2 together, are capable of interfering with JAK-STAT signaling by preventing STAT-1 phosphorylation. However, there could be other proteins of EHV-1 which also inhibit type-I IFN signaling and further studies are required to identify those proteins.

4.8 T953 Virus Mediated Inhibition of ISG Induction

Because the virus down-regulated type-I IFN production as well as signaling at later time points (12 h.p.i.), further studies were conducted to evaluate whether the virus was interfering downstream to the JAK-STAT signaling. Two important ISGs, viperin and ISG56 which have been shown to have antiviral properties against diverse group of viruses such as HCV, Dengue virus, influenza virus, HPV including herpesviruses were evaluated in the study [296, 298, 396-398]. It was tested whether T953 virus was influencing the expression of these two ISGs at either the transcriptional level or the translational level, by real-time RT-PCR assay and WB respectively.

In the absence of rEqIFN-α treatment T953 infection did not induce any viperin protein expression (Fig 3.21, 3.23) at either early or later time points although at early time points IFN-β was found. At late time points T953 also prevented poly I:C induced viperin expression. However, when viral late gene expression was blocked then T953 infection of EECs induced viperin protein expression even in the absence of any external stimuli such as poly I:C (Fig 3.21 B). These suggested that inhibition of viperin expression by T953 is viral late gene dependent.

When EECs were treated with rEqIFN-α to induce expression of viperin as well as ISG56 mRNA, T953 infection in the presence of rEqIFN-α showed a significant decrease of viperin and ISG56 mRNA when compared with mock infected cells treated with rEqIFN-α (Fig 3.22). This inhibitory effect of T953 virus on viperin and ISG56 expression was also seen at the protein level (Fig 3.23). Similar to this, HSV-1 inhibits ISG56 induction at both mRNA and protein level [399]. HSV-1 ICP0 protein is sufficient to inhibit ISG56 induction [400] although other proteins
are also possibly involved in the inhibition of ISG56 and other ISGs [399, 401]. In summary, the
data suggests that T953 infection not only inhibits type-I IFN induction but also suppresses type-I
IFN signaling and its downstream effects in vitro in EECs. By these mechanisms EHV-1 subverts
the host’s principal type-I IFN mediated immune responses which is undoubtedly related to its
success as a pathogen.

4.9 Future Scope

To the best of our knowledge, for the first time, results from this dissertation have identified
new and novel strategies that EHV-1 T953 strain adopts to subvert the host’s most important
innate antiviral immunity. This study has also identified for the first time that EHV-1 genes ORF1
and/or ORF2 have anti-IFN activity. Maa et al [106] and Soboll Hussey et al [342] also have
identified ORF1 and ORF2 as viral virulence factors. The ORF1 and ORF2 encode membrane
protein UL56 (pUL56) and membrane protein VI respectively (Table 1.1). Identification of these
virulence factors will give new insights for the design of more effective modified live virus
vaccines (MLV), by creating recombinant viruses devoid of those pathogenicity factors to control
EHV-1 infection. Future studies to identify more viral proteins which are responsible for
suppressing type-I IFN induction will also improve the quality of MLV and the control of the
infection.

This study provides the groundwork for a new prospective area in the field of EHV-1
research. Findings from this dissertation show that EHV-1 suppresses type-I IFN induction in
EECs which is a relevant cell type for the pathogenesis of EHV-1. Previous reports showed that
serum IFN levels in EHV-1 infected horses are at their peak when clinical signs are highest
suggesting that a high level of serum IFN correlates with the severity of clinical disease. We think
that EHV-1 infection causes induction of type-I IFN by other cells in the body such as PBMCs,
dendritic cells but for the benefit of virus replication, EHV-1 shuts down the production of type-I
IFN in the endothelial cells of the blood vessels after an initial transient induction. Furthermore,
EHV-1 infection also down-regulates type-I IFN signaling by suppressing STAT-1 translocation
into the nucleus from the cytoplasm and preventing subsequent ISG induction. This inhibition of ISG production makes the virus highly resistant to the effect of type-I IFN.

While this study was done using a neuropathogenic virus T953 as a model virus, to show the relationship of type-I IFNs to EHM, for comparison a non-neuropathogenic EHV-1 virus should be studied to evaluate the type-I IFN response. Although this study shows that the T953 late genes are probably responsible to suppress the type-I IFN induction, the specific viral genes or proteins were, however, not identified. Viral proteins encoded by ORF1/2 appear to be involved in the evasion of type-I IFN mediated JAK-STAT signaling. There could be other proteins also involving in this type-I IFNs evasion strategies. Further studies are required to identify those novel anti-IFN proteins. Taken together all the data, we propose the following model:

All the blocks in the type-I IFN signaling in our model may be non-specific to the downstream effect to the downregulation of IFNAR1 and IFNAR2 which was not studied.

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During natural infection in horses, endothelial cells of the blood vessels of particular targeted organs including the nasal mucosa, lungs, pregnant uterus and nervous system become infected with EHV-1. The local IFN milieu in those targeted organs might strongly influence the pathogenesis of EHV-1 disease [338]. So, another important area of study will be why endothelial cells of some targeted organs or systems of horse are particularly affected by T953 infection. Does IFN-β have any role in the EHV-1 infection process particularly in endothelial cells of some targeted organs? The answers to these questions may give us clues to identify host factors that could be responsible for EHV-1 abortions as well as EHM.
## APPENDIX I

### List of abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Aa</td>
<td>Amino acid</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>ADCC</td>
<td>Antibody dependent cell cytotoxicity</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<td>Ag</td>
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<td>AHV-1</td>
<td>Asinine herpesvirus-1</td>
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<td>Asinine herpesvirus-3</td>
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<td>AMV</td>
<td>Avian myeloblastoma virus</td>
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<td>β2m</td>
<td>β2-microglobulin</td>
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<td>BHV-1</td>
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</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>bICP0</td>
<td>infected cell protein 0 of bovine herpesvirus-1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CER</td>
<td>Cytoplasmic extraction reagent</td>
</tr>
<tr>
<td>CF</td>
<td>Complement fixing</td>
</tr>
<tr>
<td>CMC</td>
<td>Carboxy-methyl cellulose</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CTLp</td>
<td>Cytotoxic T lymphocyte precursor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>gC</td>
<td>Glycoprotein C</td>
</tr>
<tr>
<td>gD</td>
<td>Glycoprotein D</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GHV-1</td>
<td>Gazelle herpesvirus-1</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>h.p.i.</td>
<td>hour post infection</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>ICP</td>
<td>Infected cell protein</td>
</tr>
<tr>
<td>ICTV</td>
<td>International Committee on Taxonomy of Viruses</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IFA</td>
<td>Indirect immunofluorescence assay</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNAR1</td>
<td>Interferon α receptor 1</td>
</tr>
<tr>
<td>IFNAR2</td>
<td>Interferon α receptor 2</td>
</tr>
<tr>
<td>IFNGR</td>
<td>Interferon γ receptor</td>
</tr>
<tr>
<td>IFNλR</td>
<td>Interferon λ receptor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IRF-3</td>
<td>Interferon regulatory factor 3</td>
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</tbody>
</table>
IRF-7  Interferon regulatory factor 7
IR    Internal repeat
ISG   Interferon stimulated genes
ISRE  Interferon stimulated response elements
IU    International unit
JAK   Janus kinase
KyED  Kentucky equine dermis cells
L     Late
LGP2  Laboratory of Genetics and Physiology 2
LPS   Lipopolysaccharides
LU    Laboratory unit
mAb   Monoclonal antibody
MCMV  Murine cytomegalovirus
MDBK  Madin-Darby bovine kidney cells
MDA-5 Melanoma differentiation associated gene 5
MHC-I Major histocompatibility complex-I
MLV   Modified live virus vaccine
MOI   Multiplicity of infection
mRNA  Messenger RNA
MTOC  Microtubule organizing center
NER   Nuclear extraction reagent
NF-κB Nuclear factor κB
NK    Natural killer cells
NPC   Nuclear pore complex
OAS   Oligoadenylate synthetase
OD    Optical density
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAA</td>
<td>Phosphonoacetic acid</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyinosinic acid:polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PrV</td>
<td>Pseudorabies virus</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>rEqIFN-α</td>
<td>Recombinant equine interferon α</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid inducible gene I</td>
</tr>
<tr>
<td>RK-13</td>
<td>Rabbit kidney cells</td>
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<tr>
<td>RLR</td>
<td>RIG like receptor</td>
</tr>
<tr>
<td>ROCK-1</td>
<td>Rho associated coiled coil kinase-1</td>
</tr>
<tr>
<td>RSAD2</td>
<td>Radical S-adenosylmethionine domain containing 2</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SeV</td>
<td>Sendai virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCF</td>
<td>Tissue culture fluid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco-mosaic virus</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TR</td>
<td>Terminal repeat</td>
</tr>
<tr>
<td>UL</td>
<td>Unique long</td>
</tr>
<tr>
<td>US</td>
<td>Unique short</td>
</tr>
<tr>
<td>USD</td>
<td>United States Dollar</td>
</tr>
<tr>
<td>Vhs</td>
<td>Virion host shut off protein</td>
</tr>
<tr>
<td>VN</td>
<td>Virus neutralizing</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella-zoster virus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
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