Distribution Agreement

In presenting this dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my dissertation in while or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this dissertation. I retain all ownership rights to the copyright of the dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Michael K. Lo Date

Characterization of the Nipah Virus P gene Products

And the Innate Immune Responses of

Nipah Virus Infected Endothelial and Neuronal Cells

By
Michael K. Lo
Doctor of Philosophy
Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____
Paul A. Rota, Ph.D.
Advisor

_____
Richard Compans, Ph.D.
Co-Advisor

_____
Jacqueline Katz, Ph.D.
Committee Member

_____
Oscar Perng, Ph.D.
Committee Member

_____
David Steinhauer, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Characterization of the Nipah Virus P gene Products And the Innate Immune Responses of Nipah Virus Infected Endothelial and Neuronal Cells

By

Michael K. Lo

B.S., University of California, San Diego, 2001

Advisor: Paul A. Rota, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy
In Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis
2009

Abstract

Characterization of the Nipah Virus P gene Products And the Innate Immune Responses of Nipah Virus Infected Endothelial and Neuronal Cells

By Michael K. Lo

Nipah virus (NiV) is a highly pathogenic paramyxovirus which frequently causes fatal encephalitis in humans. Although the pathology of this disease is well defined, the molecular mechanisms of NiV pathogenesis are unclear. Endothelial cells and neurons are important cellular targets in the pathogenesis of this disease. The goals of this dissertation are to characterize the expression of NiV phosphoprotein (P)-derived gene products in the context of infection, and to characterize the endothelial and neuronal cell innate immune responses against NiV infection.

In this study, our sequence analysis of multiple cloned mRNAs from infected cells showed that the P gene mRNA editing frequencies of henipaviruses are higher than those reported for most other paramyxoviruses. Antisera generated against synthetic peptides from the P, V, W, and C proteins of NiV were able to detect all four proteins in NiV infected cells and in purified virions. In infected Vero cells, the W protein was detected in the nucleus while P, V, and C were found in the cytoplasm. The W protein was shown to co-immunoprecipitate with karyopherin $\alpha 3$.

Although previous plasmid expression studies showed that the expression of individual NiV P gene products can antagonize the innate antiviral response in several cell lines, it is not known whether live NiV infection of physiologically relevant cellular targets reflect the results seen in those plasmid expression studies. Little has been done

in regards to the molecular mechanisms of vasculitis seen in human cases. In this study, we characterized the growth kinetics and the innate immune responses of primary endothelial cells and a neuronal cell line. NiV infected endothelial cells produced a functionally robust IFN-β response, which correlated with a differential localization of the NiV W protein when compared with infected neuronal cells, which lacked any detectable antiviral response. We also demonstrated that NiV infection of endothelial cells induced a significant increase of inflammatory chemokines secreted into the cellular supernatant, and that these supernatants induced a corresponding increase in monocyte and T-lymphocyte chemotaxis. This study is the first *in vitro* characterization of the innate immune response against NiV infection in physiologically relevant cell types.

Characterization of the Nipah Virus P gene Products And the Innate Immune Responses of Nipah Virus Infected Endothelial and Neuronal Cells

By

Michael K. Lo

B.S., University of California, San Diego, 2001

Advisor: Paul A. Rota, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy
In Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis
2009

Table of Contents

List of Figures and Tables

Chapter 3.

Chapter 1

Literature Review

Partial contents of this chapter have been published in the Journal of Clinical Virology and Advances in Virus Research

Introduction

Nipah virus (NiV) is a paramyxovirus in the genus *Henipavirus* of the subfamily *Paramyxovirinae* within the family *Paramyxoviridae*, which is in the order Mononegavirales. The emergence of NiV and its genus counterpart Hendra virus (HeV) began in 1994. HeV causes a febrile respiratory illness in humans and animals, and was responsible for the deaths of two humans out of three infections and 17 horses in three separate incidents in Australia between 1994 and 1999 (Hooper et al., 2000; Murray et al., 1995; Selvey et al., 1995). One of the two fatal human HeV cases resulted in death 11 months post-infection (O'Sullivan et al., 1997). The first known human infections with NiV were detected during an outbreak of severe febrile encephalitis in peninsular Malaysia and Singapore from the fall of 1998 to the spring of 1999. A total of 276 patients (105 fatal) with viral encephalitis due to NiV disease were reported in peninsular Malaysia and Singapore, mostly among adult males who were involved in pig farming or pork production activities (CDC, 1999a; b; Chua *et al.*, 2000). More recently, NiV has been established as the cause of fatal human encephalitis in Bangladesh during the winters of 2001, 2003, 2004, 2005, and 2008 (ICDDRB, 2004b; c; PROMED-MAIL, 2008; WHO, 2004) as well as in India in 2001 and 2007 (Chadha et al., 2006; PROMED-MAIL, 2007). Fruit-eating bats in the order Megachiroptera are a natural reservoir for NiV and HV (Chua et al., 2002; Halpin et al., 2000; Yob et al., 2001), while humans are infected via intermediate hosts such as pigs (Amal et al., 2000; Chew et al., 2000; Paton et al., 1999), by exposure to infected fruit bats or material contaminated by infected bats (Hsu et al., 2004; ICDDRB, 2004d), or by direct human-to-human transmission (Gurley et al., 2007a). HeV and NiV cross-react antigenically, but not with any of the other

paramyxoviruses (Wang et al., 2001). Henipaviruses are the only zoonotic paramyxoviruses that are highly pathogenic in humans, but have a host range that spans five terrestrial mammalian orders, exceeding that of the morbillivirus canine distemper virus (CDV), which infects species across three orders (Appel & Summers, 1995; Chua *et al.*, 2000; Middleton *et al.*, 2002; Mohd *et al.*, 2000; Reynes *et al.*, 2005; Roelke-Parker *et al.*, 1996). No vaccines or antiviral drugs are currently available for NiV, although during the NiV outbreak in Malaysia, patients receiving ribavirin either orally or intravenously showed a lower mortality rate (Chong et al., 2001).

Epidemiology

The first known human infections with NiV were detected during an outbreak of severe febrile encephalitis in peninsular Malaysia and Singapore from the fall of 1998 to the spring of 1999. A total of 276 patients with viral encephalitis due to NiV were reported in peninsular Malaysia and Singapore, mostly among adult males who were involved in pig farming or pork production activities, with 106 fatalities (Case fatality rate (CFR)- 38.5%) (CDC, 1999a; b; Chua *et al.*, 2000; Parashar *et al.*, 2000). In spite of isolation of NiV from urine, saliva, and respiratory secretions from infected patients (Chua et al., 2001), the primary mode of transmission to humans during this outbreak was pig-to-human (Parashar et al., 2000), as the subsequent culling of over 1 million pigs lead to the eventual containment of the outbreak. Transmission among pigs occurred through direct contact with urine and mucosal secretions, as experimental infection of pigs confirmed the quick spread of NiV from infected to uninfected pigs in close contact (Middleton et al., 2002).

Since the initial outbreak, NiV has caused fatal human encephalitis in Bangladesh during 2001, 2003, 2004, 2005, 2007, and 2008 (Hsu *et al.*, 2004; ICDDRB, 2004a; c; Luby *et al.*, 2006; PROMED-MAIL, 2008; WHO, 2004) as well as in India in 2001 and 2007 (Chadha et al., 2006; Harit et al., 2006; PROMED-MAIL, 2007). These smaller outbreaks had a marked increase of CFR, which ranged from 67% to 92%. A number of significant epidemiologic features associated with these outbreaks differed from those of the initial Malaysian outbreak. In the outbreak in Meherpur, Bangladesh in 2001 (CFR-69%, 13 cases), both close contact with infected patients as well as with sick cows were associated with NiV infection, although samples from cows were not available for testing (Hsu et al., 2004). Eight households were affected, and of the 13 cases reported, 9 were related either by blood or by marriage to the index patient. Person-to-person contact was also a primary risk factor during the 2003 outbreak in the Nagoan district of Bangladesh (CFR- 67%, 12 cases), but in this instance there were no blood relationships between affected households (Hossain et al., 2008). In the outbreak in Faridpur, Bangladesh in 2004 (CFR-75%, 36 cases), person-to-person transmission was implicated, and the possibility of nosocomial transmission was demonstrated by the detection of NiV RNA on hospital surfaces (Gurley et al., 2007a; Gurley et al., 2007b). Retrospective analysis of an outbreak in Siliguri, India in 2001 (CFR-74%, 66 cases) confirmed that nosocomial transmission resulted in the amplification of the outbreak (Chadha et al., 2006). Ingestion of NiV-contaminated date palm sap was reported as the primary risk factor during an outbreak in the Tangail district of Bangladesh that spanned from December 14th 2004 through January 31st 2005 (CFR 92%- 12 cases), and is potentially linked to a more recent outbreak of NiV in the Manikganj and Rajbari districts of Bangladesh (Luby et al.,

2006; PROMED-MAIL, 2008). In summary, the distinctive epidemiological characteristics of the Bangladesh and India outbreaks were the documentation of person-to person transmission, potential food borne transmission, accompanied by higher CFR. The increase in CFR in the Bangladesh outbreaks may be due to the inherent strain-specific differences between Malaysian and Bangladesh NiV, or the comparatively lower level of supportive care available compared to Malaysia and Singapore (Harcourt et al., 2005).

Virus Reservoir

Despite the ability of NiV to infect across many mammalian species (dogs, cats, ferrets, pigs, horses), the absence of neutralizing antibody in non-infected hosts indicated that these were 'dead-end hosts'(Mohd *et al.*, 2000). Since HeV had been detected in fruit bats of the *Pteropus* genus, they were seen as the logical reservoir for NiV (Halpin et al., 2000). Neutralizing antibodies to NiV were found primarily in *Pteropus hypomenalus* and *Pteropus vampyrus* during initial surveillance studies, but virus was not isolated (Yob et al., 2001). It was not until bat urine and swabs of contaminated fruit were collected that NiV was isolated from *Pteropus hypomenalus* on Tioman Island (Chua et al., 2002). Since then, anti-NiV antibodies have been detected in other *Pteropus* species (*Pteropus lylei*, *Pteropus giganteus*,) as well as in non-*Pteropus* species (*Hipposideros larvatus*, *Scotophiilus kuhlii*, *Myotis sp.*, *Rosettus sp.*) albeit at much lower frequencies, from southern China, Cambodia, Thailand, Indonesia, Bangladesh, to Madagascar (Hsu et al., 2004; Li et al., 2008; Reynes et al., 2005; Sendow et al., 2006; Wacharapluesadee et al., 2005). More recently in separate studies, anti-Henipavirus

antibodies and Henipavirus-like RNA have been detected in the fruit bat species *Eidolon helvum* in the West African country of Ghana (Drexler et al., 2009; Hayman et al., 2008). Molecular characterization of viruses from Malaysia, Cambodia, India, and Bangladesh indicate that viruses circulating in different areas have unique genetic signatures, suggesting that these strains may have coevolved within the local natural reservoirs. The sequence diversity found among viruses isolated from various Bangladesh outbreaks suggests the possibility of multiple introductions into the human population (Pulliam *et al.*, 2005). Experimental infection of *Pteropus poliocephalus* fruit bats indicated that NiV-infected bats develop subclinical infection characterized by sporadic viral excretion via urine, seroconversion, and scant evidence of viral antigen in tissues (Middleton et al., 2007). The use of urine during mutual grooming by these particular species of fruit bats could possibly sustain intermittent transmission of virus throughout bat roosts numbering in the thousands (Hall & Richards, 2000).

Clinical presentation & pathological manifestations

NiV causes rapid acute encephalitis with a high mortality rate. The incubation period during the Malaysian outbreak ranged from 4 days to 2 months, with a majority of patients reporting within 2 weeks or less. The primary clinical features were fever, headache, dizziness, vomiting, and reduced levels of consciousness. Distinctive clinical signs included segmental myoclonus, hypertension, tachycardia, areflexia, and hypotonia (Goh et al., 2000). The direct cause of death was likely due to effects of encephalitis especially when the brainstem was affected. Patients who retained normal levels of

consciousness throughout infection were less likely to develop symptoms associated with a poor prognosis, such as abnormal doll's eye reflex and tachycardia.

During the Bangladesh outbreaks, the disease incubation period was between 6-11 days, which was markedly shorter than that of the Malaysian outbreak (Hossain et al., 2008). One clinical characteristic distinguishing the Bangladesh and Indian outbreaks from the Malaysian outbreak was the prevalence of respiratory symptoms. Only 14% of Malaysian and 27% of Singaporean patients reported respiratory distress, while close to 70% of Bangladesh and Indian patients reported these symptoms (Chadha et al., 2006; Hossain et al., 2008). A majority (90%) of cases from the 2001-2004 Bangladesh outbreaks had altered mental status, while only 21% of patients presented this sign during the Malaysian outbreak (Hossain et al., 2008). Vomiting was also a prevalent feature of Bangladesh outbreaks (58% of patients) compared with the Malaysian outbreak (27%). Clinical features associated with a poor prognosis during the Bangladesh outbreaks included having a temperature > 37.8°C, altered mental status, unconsciousness, respiratory difficulty, and abnormal plantar reflexes (Hossain et al., 2008).

From the Malaysian outbreak, it was determined that 7.5% of patients who had recovered from NiV infection developed relapse encephalitis, while 3.7% of patients who had non-encephalitic or asymptomatic infection developed late-onset encephalitis (Tan et al., 2002). Clinical features included fever, headache, seizures, and focal neurological signs. Patients who experienced relapsed or late-onset encephalitis had a lower mortality rate (18%) than those who suffered acute Nipah encephalitis (40%). However, those with relapse or late-onset encephalitis tended to have worse residual neurological deficits (61%) than those who had acute encephalitis (22%). Outcomes of those patients having

additional neurological episodes include ataxia, cognitive impairment, dysphasia, pseudobulbar palsy, tetraparesis, nystagmus, epilepsy, and death. Magnetic resonance imaging (MRI) displayed patchy regions of confluent cortical lesions mainly in the cerebral hemisphere (Tan et al., 2002). This delayed-onset neurological feature has been documented in for Hendra virus (HeV) infection, as well as in subacute sclerosing panencephalitis (SSPE) caused by measles virus (Bojinova et al., 2004; Dubois et al., 2005; O'Sullivan et al., 1997). MRI of measles SSPE patients displayed similar diffuse cortical and subcortical hyperintensities found in these late-onset cases of NiV encephalitis (Ozturk et al., 2002; Sener, 2004).

Twenty-two patients who recovered from NiV illness in Bangladesh were examined via MRI and neurologic questionnaire to determine the extent of long-term neurologic and functional outcomes (Sejvar et al., 2007). Among this group, those who suffered acute encephalitis frequently developed neurological sequelae including encephalopathy, cranial nerve palsies, and dystonia. While most of the MRI findings agreed with previous observations found in relapse/late-onset encephalitis patients, there was one patient who had confluent abnormalities during acute infection. This demonstrated that the confluent and diffuse cortical involvement typically shown in MRIs of relapse/late-onset patients could be found in early NiV infection. This confirmed a smaller study in which MRI findings of 4 acute NiV encephalitis patients showed confluent high signal lesions in both gray and white matter (Quddus et al., 2004). Taken together, the clinical presentation of cases from Bangladesh and India significantly differed from Malaysian cases.

A systemic multi-organ vasculitis associated with infection of endothelial cells was the main pathologic feature of NiV infection, with infection being most pronounced in the central nervous system (CNS) (Wong et al., 2002). In the vascular endothelium, immunohistochemical analysis showed intense staining of endothelial, parenchymal, and multinucleate giant cells characteristic of paramyxovirus infections. However, evidence of endothelial infection and vasculitis was also observed in other organs, including lung, heart, spleen, and kidney. NiV has been isolated from cerebrospinal fluid, tracheal secretions, throat swabs, nasal swabs, and urine specimens of patients (Chua et al., 2001; Goh et al., 2000; Wong et al., 2002). NiV infection can also become a chronic condition, as serious neurological disease can occur late (more than 4 years) following a non-encephalitic or asymptomatic infection (Chong & Tan, 2003; Tan *et al.*, 2002; Wong *et al.*, 2002).

The disease manifestations of both natural and experimental Henipavirus infections vary greatly between its natural chiropteran hosts and other susceptible terrestrial species. In Pteropid fruit bats, the infection is subclinical with low levels of detectable antigen, only rarely causing vasculitis in select organs (Hooper et al., 2001; Middleton et al., 2007; Williamson et al., 1998; Williamson et al., 2000). In contrast, among terrestrial species systemic vasculitis is the predominant finding particularly in the pulmonary and nervous systems, with virus readily being recovered from nasal secretions, urine, and internal organs (Daniels *et al.*, 2001; Hooper *et al.*, 2001). Due to their high pathogenicity and lack of therapeutics, NiV and HeV are classified as biosafety level (BSL) 4 agents. Given that very few laboratories have BSL-4 capability, data from experimental infections is limited. Infection of golden hamsters with NiV reproduces the

pathogenesis of acute NiV infection in humans, and could provide an animal model for studying the pathogenesis of NiV. Infection by the intranasal or intraperitoneal routes caused a fatal neurological disease associated with severe pathological lesions in the hamster brain. Viral antigen and RNA were detected in endothelial cells, neurons, lung, kidney, and spleen, while virus and/or viral RNA could be recovered from most organs and urine, but not serum (Georges-Courbot et al., 2006; Guillaume et al., 2006). Cats, guinea pigs, and ferrets have also been tested as potential models for NiV infection (McEachern et al., 2008; Pallister et al., 2009; Torres-Velez et al., 2008; Weingartl et al., 2005).

Classification and Morphology

NiV belongs to the family *Paramyxoviridae*, and has a non-segmented, negative-stranded (NNS) RNA genome consisting of helical nucleocapsids encased in an envelope forming relatively spherical, pleomorphic virus particles. There are two subfamilies within the family *Paramyxoviridae*, the *Paramyxovirinae* and the *Pneumovirinae*. The subfamily *Paramyxovirinae* is divided into 5 genera: Rubulavirus (prototype, mumps virus), Respirovirus (prototype, human parainfluenza virus 1), Morbillivirus (prototype, measles virus), Avulavirus (prototype Newcastle disease virus), and with the emergence of NiV and its counterpart Hendra virus (HeV), a new genus Henipavirus (Bellini *et al.*, 1998).

Electron microscopy studies using both negative stain and thin section preparations depicted extracellular NiV virions as tangled collections of filamentous, helical nucleocapsids encased by the viral envelope (Chua et al., 2000; Goldsmith et al.,

2003). While NiV virions share common morphological characteristics with the rest of the family *Paramyxoviridae* (i.e., herringbone-like nucleocapsid structure, nucleocapsid accumulation along plasma membranes during budding), there were several distinct characteristics observed. There were unusual cytoplasmic ring-like reticular inclusions (RI) found in proximity to endoplasmic reticulum membranes. These inclusions were distinct from the typical nucleocapsid inclusions (NCI) found in paramyxovirus-infected cells. There were also long cytoplasmic tubules found in the periphery of NiV infected cells, which were occasionally incorporated into virus particles. NiV virions were on average larger in diameter (500 nm) than typical paramyxoviruses (150-400 nm), with extreme variations in size (180nm-1900nm) (Goldsmith et al., 2003). There are subtle ultrastructural differences that distinguish NiV and HeV. When viewed by negative-contrast electron microscopy, the surface projections of HeV virions have a double-fringed appearance, while those of NiV have a single fringe (Hyatt et al., 2001). The localization of the cell nuclei and viral nucleocapsids within infected cells also differentiates NiV and HeV. Along with the viral nucleocapsids, the cell nuclei of NiV-infected cells localize to the cell periphery. In HeV infected cells, both the cell nuclei and HeV nucleocapsids are either more centrally located or randomly distributed in the cytoplasm (Hyatt et al., 2001). In situ hybridization studies using both positive and negative sense probes in NiV-infected cell culture and brain specimens taken from fatal NiV infections indicated that unencapsidated viral mRNA is almost exclusively found in the novel reticular inclusions (RI) mentioned previously, and not in the NCIs (Goldsmith et al., 2003). Given that the RI complexes were found in the context of a natural

infection, these results suggest the possibility that for NiV, the newly described RI structures are an integral part of the transcription and replication process.

The Genome Organization of NiV

The NiV genome consists of six genes encoded from 3' to 5', the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment glycoprotein (G), and large (L) polymerase, respectively. The genome of NiV is 18,246 nucleotides (nt) long, making it significantly larger than most other paramyxoviruses, with the exception of the rodent-borne J-virus and Beilong virus (Figure 1) (Jack *et al.*, 2005; Li *et al.*, 2006; Magoffin *et al.*, 2007b). The large genome size is due to a longer open reading frame (ORF) encoding the phosphoprotein (P) and to the large 3' untranslated regions (UTR) that flank each gene with the exception of the large polymerase gene (Harcourt et al., 2001; Harcourt et al., 2000; Mayo, 2002; Wang et al., 1998; Wang et al., 2000a; Yu et al., 1998a; Yu et al., 1998b). The overall nucleotide homology between the viral genomes of the Malaysian and Bangladeshi strains (from 2004) is 91.8%, with nucleotide homologies being higher in the protein coding regions than in the non-coding regions (Harcourt et al., 2005).

The organization of the NiV genome indicates that it likely has a similar replication strategy to other paramyxoviruses, which was demonstrated by the requirement of the N, P, and L proteins for mini-genome replication (Halpin et al., 2004; Harcourt et al., 2001; Harcourt et al., 2000; Wang et al., 2000b). The genus-specific 3' leader (55 nt for NiV) and 5' trailer (30 nt for NiV) regions of paramyxovirus genomes serve as promoters for both transcription and replication located at the ends of the

genome (Lamb & Parks, 2007). The coding sequence of each gene is flanked by untranslated regions (UTRs) that contain a gene-start (GS) sequence and a gene-end (GE) sequence. Immediately following the GE sequence is a short non-coding intergenic region (GAA in NiV) that precedes the gene-start sequence of the following gene. Studies of other paramyxoviruses have shown that GE signals direct polyadenylation and termination of each mRNA, while GS signals direct initiation and capping (Lamb & Parks, 2007). The terminal 12-13 nucleotides of the genome and antigenome are identical, which indicates their critical role as replication promoters. Minigenomes of paramyxoviruses, which incorporate the leader, the GS of the N protein, a reporter gene, the GE of the L protein, and the trailer have been developed for many paramyxoviruses including NiV (Chattopadhyay *et al.*, 2004; Durbin *et al.*, 1997; Halpin *et al.*, 2004; Hausmann *et al.*, 1996; Jiang *et al.*, 2008; Keller *et al.*, 2001; Magoffin *et al.*, 2007b; Perez *et al.*, 2003; Raha *et al.*, 2004; Rosario *et al.*, 2005; Sidhu *et al.*, 1995). A fine mapping study of the NiV antigenomic promoter used a T7 polymerase-driven NiV minigenome system to determine the precise boundaries of the *cis*-acting signals contained therein (Walpita & Peters, 2007). The results of the study indicated a bipartite promoter structure, with two critically conserved regions (nt 1-12, and 79-91). The structure of the internal antigenomic promoter element (nt 79-91), while analogous in position to its equivalent in the Sendai virus antigenomic promoter contained a distinct 5’-(GNNNUG)$_{14\text{-}15}$(GNNNNN)$_{16}$ motif (Tapparel *et al.*, 1998; Walpita & Peters, 2007).

General Overview of the NiV Replication Cycle

NiV virions adhere to host cell surfaces through the recently identified receptor Ephrin-B2, which is expressed on neurons, smooth muscle, and endothelial cells surrounding small arteries (Bonaparte et al., 2005; Negrete et al., 2005). Ephrin-B3, a related molecule with less affinity for NiV G, has nonetheless also been found to serve as a functional alternative receptor for NiV (Negrete et al., 2006). It has also recently been shown that NiV can enter cells via macropinocytosis (Pernet et al., 2009).

After receptor binding by NiV G, the F protein then mediates fusion between the viral envelope and the host cell membrane and the viral ribonucleocapsid is then released into the cytoplasm (Lamb & Parks, 2007). Upon ribonucleocapsid release into the host cell cytoplasm, transcription of viral mRNA begins, with the encapsidated genome serving as the template for mRNA synthesis. The polymerase complex (P & L proteins) initiates transcription at the promoter region in the 3' leader. Each gene is transcribed, being regulated by transcriptional start and stop sites at their respective regions in the gene. The polymerase is thought to "stutter" at the transcriptional stop signals in order to synthesize poly A tails of the mRNA. The polymerase pauses at the gene stop signal, crosses the intergenic region, and reinitiates transcription at the next gene start signal. During this process, some of the polymerase complexes will fall off from the template. Given that there is only one promoter at the 3' leader, these polymerase complexes can only reinitiate transcription at the 3' end. This results in a differential gradient of transcription, in which the genes closer to the 3' end are transcribed more often than genes closer to the 5' end of the genome (Bellini *et al.*, 1998).

As translation of viral mRNA occurs, viral proteins accumulate in the cell, and an unidentified mechanism triggers the polymerase to stop transcription and initiate genome

replication. During the genome replication process, the polymerase complex enters the 3' genomic promoter in a similar manner as during transcription, but ignores the mRNA transcriptional signals at gene junctions, causing the polymerase to transcribe a full-length positive-sense antigenome. A promoter in the 3' non-coding region of the antigenome serves as the location where synthesis of new, negative-sense genomic RNA begins. Newly-made genomes can in turn, serve as templates for transcription (Lamb & Parks, 2007).

In order to assemble virus particles, the genome replication process must be terminated, newly made genomes must be properly encapsidated, and regions of the cell membrane need to be prepared to accept budding nucleocapsids (Lamb & Parks, 2007). Polymerase complexes stay associated with packaged nucleocapsids and are responsible for the next round of infection. The glycoproteins are synthesized in the endoplasmic reticulum (ER) and mature through the Golgi network to the cell membrane. The NiV F glycoprotein is a unique exception to the rule, as F protein processing and maturation occurs in the endosome (Diederich et al., 2005). Cytoplasmic tails of the F and G glycoproteins play a role in the interaction with the M protein, which is the protein most likely responsible for driving virus particle assembly and budding (Ciancanelli & Basler, 2006; Lamb & Parks, 2007; Ong *et al.*, 2009; Patch *et al.*, 2007; Patch *et al.*, 2008; Sanderson *et al.*, 1994).

Since its emergence, numerous studies have provided insight on the molecular characteristics of NiV genes as well as the mechanistic details of NiV infection, which include virus entry, protein localization and processing, genome replication, and virus particle assembly.

NiV Membrane Proteins

Viruses in the subfamily *Paramyxovirinae* encode two integral membrane glycoproteins: an attachment glycoprotein (HN, H, or G) and a fusion (F) glycoprotein. Previous studies utilizing recombinant vaccinia viruses individually expressing F and G proteins of both HeV and NiV indicate that each protein induces neutralizing antibodies, and are both required to mediate cell fusion (Bossart et al., 2001; Tamin et al., 2002).

NiV G glycoprotein

The NiV G protein is a 602 amino acid type II membrane protein that forms tetramers upon cell surface expression, and serves primarily in cellular receptor binding (Levroney et al., 2005; Wang et al., 2001). In contrast to the attachment glycoproteins of other paramyxoviruses, NiV G lacks both hemagglutinin and neuraminidase activities (Tamin et al., 2002). While it had long been thought that NiV utilizes a protein receptor instead of a carbohydrate moiety for virus entry (Eaton et al., 2004), this theory has only recently been experimentally confirmed by two independent groups using contrasting approaches (Bonaparte et al., 2005; Negrete et al., 2005). One approach utilized a recombinant soluble immunoadhesin consisting of the ectodomain of the NiV G fused with the Fc region of human IgG1 to immunoprecipitate cell surface proteins from NiV fusion-permissive cells. Trypsin digestion and mass spectrometric analysis of the immunoprecipitated surface proteins identified only 1 transmembrane protein, confirmed by 2 independent tryptic fragments to be ephrinB2. Transfection of non-permissive cell lines with ephrinB2 rendered them permissive to both NiV-envelope-mediated fusion as well as to NiV infection, which was evaluated by using VSV-RFP viruses pseudotyped

with the NiV F and G proteins (Negrete et al., 2005). The second approach utilized microarray analyses of both permissive and non-permissive cell lines to NiV infection in order to identify plasma membrane proteins differentially expressed in permissive cell lines. From the pool of potential receptor-encoding genes identified, only ephrinB2 was able to rescue NiV-mediated cell fusion when transfected into the non-permissive cell line, as well as to render those cells permissive to NiV infection (Bonaparte et al., 2005). The expression pattern of ephrinB2 in neurons, smooth muscle, and endothelial cells correlates with the cellular tropism of NiV during infection. EphrinB2 is involved in embryogenic development, vasculogenesis, and axonal guidance (Palmer & Klein, 2003; Poliakov *et al.*, 2004). Recent studies of the requirement for ephrinB2-mediated NiV infection of endothelial cell lines underscores the correlation of this receptor with observed pathogenesis in human cases (Erbar *et al.*, 2008; Thiel *et al.*, 2008). Not long after the delineation of ephrinB2 as the primary cellular receptor for NiV infection, ephrinB3 was identified as an alternative cellular receptor for NiV infection (Negrete et al., 2006). A comparison of the respective binding affinities of ephrinB2 and ephrinB3 to a soluble form of NiV G showed that despite a 10-fold lower binding affinity than ephrinB2, ephrinB3 bound to NiV G at subnanomolar affinities. In addition, this study identified two critical residues shared between ephrinB2 and ephrinB3 that both mediate NiV entry and are crucial to their function as EphB receptor ligands (Negrete et al., 2006).

A following study by the same group identified and distinguished functional residues required for ephrinB2 and ephrinB3 interactions with NiV G. While NiV and its genus counterpart Hendra virus (HeV) share the use of ephrinB2 as a cellular receptor,

this study demonstrated a greater ability of NiV G to bind ephrinB3 than that of HeV G. By interchanging various homologous regions of NiV and HeV G proteins, they were able to map the determinant of ephrinB3 binding to a single residue that could confer equivalent ephrinB3 binding efficiency when incorporated into HeV G (Negrete et al., 2007). The authors hypothesize that the difference in the efficiency of ephrinB3 usage between NiV and HeV may be reflected in the increased neurotropism of NiV compared to HeV in human infections.

The crystal structures of the ephrinB2 and ephrinB3 interactions with NiV G have recently been determined (Bowden et al., 2008b; Xu et al., 2008). The data from both of these studies confirm the accuracy of the functional mapping performed by Negrete and colleagues, and also provides a comprehensive analysis of critical residues composing the hydrophobic binding cleft in NiV G which interacts with high affinity to ephrinB2 and ephrinB3. Further analysis of the unbound form of NiV G protein by crystallography and mass spectrometry indicated that NiV G contains highly processed complex-type glycans with negligible amounts of oligomannose-type glycans. Interestingly, the *N*-acetylglucosamine (GlcNAc) β1→ 2Man terminal structures on NiV G were noted as a potential ligand for LSECtin, a C-type lectin expressed on sinusoidal endothelial cells of lymph nodes and liver (Bowden et al., 2008a).

Most recently, a receptor binding activation site in the stalk region of NiV G was shown to trigger fusion by the F protein (Aguilar et al., 2009). This finding is consistent with observations of other paramyxovirus attachment glycoprotein interactions with their corresponding fusion proteins (Corey & Iorio, 2007; 2009; Lamb & Parks, 2007; Lee *et al.*, 2008; Melanson & Iorio, 2004; 2006). In summary, the progressive analyses of NiV

G interactions with its cellular receptors as well as its interaction with NiV F will facilitate the development of antiviral therapeutics which target these interactions.

NiV F glycoprotein

The NiV F protein is a 546 amino acid type I transmembrane protein that forms trimers on the cell surface, and is responsible for mediating the fusion of virion and host cell membranes during virus infection (Bossart et al., 2001; Levroney et al., 2005; Michalski et al., 2000; Tamin et al., 2002; Wang et al., 2001). Similar to other paramyxoviruses, activation of NiV F fusion depends on specific sequences in its cytoplasmic tail (Aguilar et al., 2007; Tong et al., 2002; Waning et al., 2004). The F proteins of paramyxoviruses are synthesized as inactive precursors called F0, and are then typically cleaved by a cellular protease to yield the biologically active F1 and F2 subunits which are linked by disulfide bonds (Lamb & Parks, 2007). The membrane-anchored subunit F1 contains a new hydrophobic amino terminus that is highly conserved, often referred to as the fusion peptide. F proteins with a multi-basic consensus sequence (R-X-R/K-R) are activated by ubiquitous host cell proteases such as furin, while F proteins with only 1 basic residue at the site of cleavage are activated by extracellular trypsin-like proteases upon transport of inactive F0 precursors to the cell membrane. Viruses with multi-basic sequences in their F protein usually cause systemic infections, while those with single basic cleavage sites such as Sendai virus cause localized infections (Klenk & Garten, 1994). A confounding observation was that despite the systemic nature of NiV infections, the cleavage site of the NiV F protein only contains one basic arginine reside at the cleavage site, unlike the multi-basic furin-like

protease consensus sequences found in most morbilliviruses, rubulaviruses, and pneumoviruses. A comprehensive mutagenesis study of the cleavage site demonstrated that the single arginine reside at the cleavage site was not required for F processing, which indicated that the protease responsible for processing F was neither trypsin nor any other furin-like protease (Moll *et al.*, 2004a). Furthermore, the absence of serum proteases did not inhibit NiV in cell culture, which implicated an intracellular protease to be responsible for F protein processing (Diederich et al., 2005). More recently, it was shown that the requirements for NiV F protein cleavage are significantly different from other ortho or paramyxoviruses, as incorporation of a multi-basic furin cleavage site in place of the wild-type cleavage site in the NiV F protein completely abolished cleavage (Diederich *et al.*, 2009).

By tracking the route of the F and G in NiV infected cells, it was determined that both proteins undergo endocytosis (Vogt et al., 2005). While the endocytosis of G occurred at a similar rate to that of typical membrane turnover and did not accumulate inside cells, it was shown that intracellular F protein accumulated over time. When site directed mutagenesis was performed on the classical endocytosis signal (Y-X-X-ϕ) contained in the F protein, not only was endocytosis abrogated, but the ability of F to mediate fusion when co-expressed with G was also ablated (Vogt et al., 2005) (Figure 2). The same group determined that F processing did not occur in any stage of the secretory pathway, but occurred in acidified endosomes (Figure 2) (Diederich et al., 2005). Eventually, it was shown that the endosomal protease cathepsin L was responsible for NiV F protein processing by using cysteine protease inhibitors and cell lines deficient in cathepsin L (Pager et al., 2006).

In order to determine whether NiV utilizes pH-dependent proteolysis for viral entry, a recent study used NiV to infect cells pre-treated with either an inhibitor of endosomal acidification (NH_4Cl) or a variety of endocytosis or cathepsin inhibitors (Diederich *et al.*, 2008). None of the inhibitors affected NiV infection. However, when cells were treated with either cathepsin inhibitors or NH_4Cl after NiV infection, there was a significant decrease of infectious virus released from the cells. This study demonstrated that cell-free NiV virions possess adequate amounts of proteolytically cleaved F protein to mediate viral fusion with the host membranes, and that the lowering of endosomal pH is only required for the maturation of new virion particles produced from an infected cell and not for viral entry (Diederich *et al.*, 2008).

It has been shown for many viral glycoproteins that N-glycans are crucial structural components that facilitate proper folding and transport (Doms *et al.*, 1993; McGinnes *et al.*, 2001; Roberts *et al.*, 1993; Segawa *et al.*, 2000; Tamura *et al.*, 2002; Zimmer *et al.*, 2001). The NiV F protein contains 5 potential glycosylation sites, 3 located in the F_2 subunit, and 2 in the F_1 subunit. The influence of N-linked oligosaccharides on the processing and biological activity of the NiV F protein has been studied by two independent groups, yielding some similar and some contrasting results. Moll and colleagues determined that 4 out of the 5 potential sites were glycosylated (N67, N99, N414, & N464), with complex glycosylation at all but the N464 site, which is of the high mannose variety. Site-directed mutagenesis of each glycosylation site demonstrated that both the amino acid sequence and the glycosylation of the N414 site were required for proper F protein processing and cell surface expression. It was shown that while deglycosylation negatively affected the overall cell surface expression of the F

mutants, it increased the fusogenic ability of the individual N414 and N464 mutants, and one N67/N99 double mutant F (Moll *et al.*, 2004b). Another a study done by an independent group confirmed both the glycosylation pattern of NiV F and the corresponding increase in fusion ability with deglycosylation. In contrast to the first study however, it was determined that individual F mutants at the N99 and N414 positions significantly increased fusogenicity (Aguilar et al., 2006). It is worth noting the difference in mutagenesis strategies employed by these two groups, which may explain the differences in their findings. While Moll and colleagues mutated the third residue of the glycosylation motif (N-X-**S/T**) (underlined), Aguilar and colleagues directly mutated the glycosylated arginine residue to glutamine (**N**-X-S/T) (underlined). This latter study tested multiple combinations of N-glycosylation mutant F proteins, and demonstrated a synergistic effect of multiple N-glycans in reducing fusion. A select panel of these mutant F proteins was used with wild-type NiV G to pseudotype VSV reporter viruses. These reporter viruses were used to evaluate the influence of F protein N-glycans on the efficiency of viral entry. The results indicated that the mutants which showed a hyperfusogenic phenotype also enhanced viral entry. This study also demonstrated that N-glycans can protect NiV from neutralizing antibodies, and that reducing the glycosylation on the F protein decreases the avidity of its interaction with the G protein (Aguilar et al., 2006).

NiV M protein

The NiV matrix (M) protein is a 352 amino acid protein, and like other paramyxovirus M proteins, is very basic with a pI of 9.99 and a charge of +12 at neutral

pH (Wang et al., 2001). The paramyxovirus M proteins provide rigidity and structure to the virion through its interactions with the cytoplasmic tail of the F protein, the ribonucleoprotein complex, and the inner surface of the virion envelope (Lamb & Parks, 2007; Sanderson *et al.*, 1994; Tashiro *et al.*, 1996; Tashiro *et al.*, 1993). Late domains motifs found in numerous viral matrix proteins recruit specific host factors to viral assembly sites where they facilitate virus release (Freed, 2002). There are several different late domain motifs found among negative stranded non-segmented viruses: P(T/S)AP, PPxY, $YP(x)_nL$, and more recently ϕ-P-x-V(Lamb & Parks, 2007). Several paramyxovirus M proteins have been shown to recruit cellular host factors to facilitate viral budding by way of these late domains (Ciancanelli & Basler, 2006; Irie *et al.*, 2007; Patch *et al.*, 2008; Schmitt *et al.*, 2005).

Ciancanelli & Basler showed that eukaryotic plasmid expression of the M protein alone can yield virus-like particles (VLP), and demonstrated the first evidence of late domain-like activity in the NiV M protein by mutating the YMYL motif in the N-terminal portion of M. Mutation of the first and fourth residues of this motif severely decreased the presence of M in the culture supernatant, and skewed the subcellular localization of the M towards the nucleus. By inserting the YMYL motif into a late domain-deficient Ebola virus VP40 matrix protein, the ability of VP40 to bud and form VLPs was restored. This study demonstrated the ability of NiV M to self-associate, and that the M proteins were enclosed by a lipid membrane which protected them from proteolysis (Ciancanelli & Basler, 2006).

A following study done by Patch and colleagues identified a potentially different late domain motif YPLGVG also in the N-terminal end of NiV M. Similar to the

previous study, it was shown that mutagenesis of this sequence either ablated or drastically decreased VLP budding, and also skewed the NiV M subcellular localization to the nucleus. However, substitution of the Ebola virus late domain with this motif along with several surrounding residues only restored the filamentous cell morphology phenotype of cells expressing Ebola VP40, and did not result in any budded Ebola VLPs (Patch et al., 2008). The authors also tested whether NiV M required VPS4A to form VLPs. VPS4A is a component of the vacuolar sorting protein (VPS) pathway that facilitates the formation of multivesicular bodies, which is a process similar to viral budding (Lamb & Parks, 2007). It was shown that co-expression of NiV M with either a wild-type or a dominant negative form of VPS4A did not affect the ability of NiV M to form VLPs. These results indicate potentially novel cellular components utilized by NiV to allow egress from an infected cell (Patch et al., 2008).

The Ribonucleoprotein Complex

The ribonucleoprotein (RNP) complex of paramyxoviruses consists of an N protein-encapsidated genome and the viral RNA-dependent RNA polymerase complex formed by the L and P proteins (Lamb & Parks, 2007). The L protein presumably possesses all the enzymatic activities for viral RNA synthesis, while the P protein serves as a scaffold between the L and the encapsidated genome (Bankamp *et al.*, 1996; Carsillo *et al.*, 2006; Harty & Palese, 1995; Houben *et al.*, 2007; Liston *et al.*, 1997).

NiV N protein

The N protein of NiV is a 532 amino acid protein responsible for encapsidating the viral genome, and also for the common herringbone-like structures seen in electron micrographs of paramyxoviruses (Lamb & Parks, 2007). Paramyxovirus N proteins typically contain two domains, with the amino-terminal domain responsible for specific interactions with the RNA genome and other N proteins, and the carboxy-terminal domain being responsible for interactions with the P protein (Egelman et al., 1989). The N proteins of the subfamily *Paramyxovirinae* are thought to cover exactly six nucleotides, which results in maximal viral replication when the genome length is divisible evenly by 6. This concept, coined "the rule of six" has been demonstrated for several paramyxoviruses, including NiV (Calain & Roux, 1993; Halpin *et al.*, 2004; Hausmann *et al.*, 1996; Kolakofsky *et al.*, 1998). As with other paramyxoviruses, NiV N has an inherent ability to assemble itself, as the expression of NiV N protein in the absence of other viral proteins results in the formation of nucleocapsid-like structures (Tan et al., 2004). A detailed mapping study of the precise regions required for NiV N self-assembly determined that the minimal contiguous sequence of N required was from amino acids 30-404, while the C-terminal 128 amino acids were dispensable (Ong et al., 2009).

The viral polymerase associates with the encapsidated by way of interaction between the P and the N proteins of the ribonucleocapsid. Studies of Sendai virus (SeV) have demonstrated the N-P interactions *in vitro* (Horikami et al., 1992). Upon mixture, individually formed complexes of N-P and P-L of different paramyxoviruses have the ability to support replication of defective interfering virus in vitro (De *et al.*, 2000; Errington & Emmerson, 1997). Also, the SeV P protein has been shown to interact with

unassembled N protein (N0) during the nascent chain assembly step of genome replication, with P serving as a protein chaperone for N by preventing non-specific aggregation of N (Curran *et al.*, 1995). The domains responsible for the N-P interaction have been determined for many of the paramyxoviruses, including NiV and HeV (Bankamp *et al.*, 1996; Cevik *et al.*, 2004; Chan *et al.*, 2004; Harty & Palese, 1995; Houben *et al.*, 2007). It was shown that the N and the P proteins of NiV and HeV were able to form both homologous and heterologous N-P complexes. In addition, it was determined that there was more than one P-binding site on the N protein, with one in the extreme carboxy-terminal region being sufficient to interact with the carboxy-terminal portion of the P protein (Chan et al., 2004).

NiV L protein

The L protein of paramyxoviruses is the largest and the least abundant viral protein found in an infected cell. The large size of the protein reflects the many different enzymatic functions attributed to it, including initiation, elongation and termination of both mRNA transcription (capping, methylation, and polyadenylation of mRNA) and genome replication (Lamb & Parks, 2007). A comparative study done on L proteins of NNS RNA viruses lead to the identification of six similar domains shared among them (Stec *et al.*, 1991). The L protein of NiV contains all six conserved domains found in NNS viruses, but the GDNQ motif found in most NNS RNA viruses is replaced by GDNE. This motif is the proposed active site where new ribonucleotides are added to a growing strand of RNA polymerized by the L protein. The mutation of the GDNQ motif to GDNE for both the rabies and rinderpest viruses resulted in significantly reduced

polymerase activities (Chattopadhyay *et al.*, 2004; Schnell & Conzelmann, 1995). Site directed mutagenesis of the glutamate residue in the motif GDNE (underlined) of the NiV L protein was recently performed (Magoffin *et al.*, 2007a). In this study, the E residue was substituted with Q, K, P, D, A, N, I, or G residues, and the resulting mutant L proteins were tested for their function in a NiV minigenome assay which measured green fluorescent protein expression (GFP) as an indicator of genome replication. It was determined that the GDNQ substitution did not significantly affect the function of NiV L, while the GDNK and GDNP mutants completely abolished GFP expression. The activity of the GDND, GDNA, GDNN, GDNI, and GNDG L protein mutants were significantly reduced compared to the wild-type GDNE and GDNQ, retaining 2-40% of wild-type L activity. The GDNQ motif that is conserved in the L proteins among many negative strand non-segmented RNA viruses has been thought to be essential to the catalytic activity of the polymerase (Poch et al., 1989). The ability for the NiV L protein to tolerate so many different substitutions in the GDNE motif at the E residue without completely losing its activity suggests that the amino acid residue at the E position is important for maintaining the overall protein structure rather than for its catalytic activity (Magoffin *et al.*, 2007a).

NiV P protein and additional P gene products

The P protein of NiV is a 709 amino acid protein, and is the only essential gene product encoded by the P gene for genome replication (Halpin et al., 2004). An early study utilizing mass spectrometry described only serine phosphorylation sites of NiV P, in contrast to both serine and threonine phosphorylation sites in HeV P (Shiell et al.,

2003). A study of the N-P protein interaction domains showed that there were at least two independent N-binding sites in the P protein; one was at the N-terminus (amino acids 3-220), while the other was at the end of the C-terminus (amino acids 636-709) (Chan et al., 2004).

A common characteristic among viruses in the *Paramyxovirinae* subfamily is the ability to generate multiple proteins from the P gene that are distinct from the P protein (Lamb & Parks, 2007). These additional gene products are usually not required for virus replication *in vitro*, though they often serve as virulence factors *in vivo* (Devaux & Cattaneo, 2004; Devaux *et al.*, 2008; Garcin *et al.*, 1997; Garcin *et al.*, 1999; Kato *et al.*, 1997a; Kato *et al.*, 1997b; Kurotani *et al.*, 1998; Patterson *et al.*, 2000; Tober *et al.*, 1998; Toth *et al.*, 2009; Valsamakis *et al.*, 1998; von Messling *et al.*, 2006). There are a number of mechanisms by which these accessory gene products are generated.

The first mechanism is by a process of non-templated co-transcriptional editing, otherwise known as RNA editing. The viral polymerase complex incorporates non-templated guanosine residues into a specific editing site of the P gene mRNA, which creates frameshifts that result in the translation of different open reading frames (ORFs) downstream of the editing site (Curran *et al.*, 1991). RNA editing is responsible for generating the viral mRNA transcripts of V and W, which share a common amino terminus with the P protein, but have respectively unique carboxy termini. The V transcript is created by the addition of 3n+1 guanosine residues to the editing site, effectively generating a +1 ORF distal to the editing site. The W transcript is generated by adding 3n+2 guanosine residues, which results in a +2 ORF distal to the editing site. The exception to this rule is for the genus Rubulavirus, in which the P protein is made

when 2 guanosine residues are added, and the V protein is made when there are no insertions in the mRNA editing site (Lamb & Parks, 2007).

A second mechanism which is responsible for expressing the C proteins is a process by which host cell ribosomes read through the original P gene mRNA start codon to use an alternative downstream translation initiation sequence (Curran & Kolakofsky, 1988a; b; 1989; Latorre *et al.*, 1998). This results in translation of a protein using a distinct overlapping ORF. Two other mechanisms include internal ribosomal binding sites, and the use of non-AUG start codons (Bellini *et al.*, 1998; Mehdi *et al.*, 1990). The P gene from certain members of the *Paramyxovirinae* subfamily (SeV) can make up to 7 non-structural accessory proteins (Curran & Kolakofsky, 1988a; b; 1989; Latorre *et al.*, 1998).

The P gene of NiV encodes three non-structural proteins (C, V, and W) in addition to the P protein. The V and W proteins are generated by RNA editing, while the C protein is encoded by a second ORF that initiates 23 nt downstream of the translational initiation site for the P ORF (Harcourt et al., 2000). Experiments using transfected plasmid vectors expressing NiV P, V, and W indicated that the V and P proteins are localized in the cytoplasm, while the W protein is in the nucleus (Rodriguez *et al.*, 2004; Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). However, it was not until recently that the mRNA editing frequency of the NiV P gene and the subcellular localization of the C, V, and W proteins were characterized in NiV infected cells (Lo et al., 2009). In this study, poly-A-plus purified RNA extracted from cells infected with one of two strains of NiV (Malaysia or Bangladesh) or its genus counterpart HeV was reverse-transcribed. The complete P gene was amplified from the cDNA by PCR, and was cloned into bacteria.

Over 100 bacterial colonies were sequenced across the RNA editing site each for NiV Malaysia, NiV Bangladesh, and HeV to determine the frequency and numbers of guanosine residue insertions at the editing site. For all three viruses tested, the P gene mRNA frequencies indicated that approximately two-thirds of all P gene transcripts were edited and 50% of all transcripts encoded for P, 25% for V, and 25% for W. Western blots performed against NiV infected cell lysates using specific anti-peptide sera against the unique regions of the P, V, W, and C proteins indicated robust expression levels of V and W, while the expression of C was markedly lower. The V, W, and C proteins were surprisingly also detected in significant quantities in sucrose gradient purified virions. Immunofluorescence assays performed on NiV infected cells using the aforementioned antisera showed distinct subcellular localizations of the P, C, V, and W proteins (see Chapter 2). The P protein, while localized in the cytoplasm, concentrated near the plasma membrane, which is in contrast to the homogenous distribution of plasmid-expressed P. The V protein was evenly distributed throughout the cytoplasm and the W protein exclusively localized to the nucleus. The C protein was distributed in the perinuclear areas in a punctuate pattern. Co-immunoprecipitation experiments from NiV infected cell lysates showed that the importin karyopherin-α3 co-precipitated with the W protein, which confirm the findings from earlier plasmid expression studies (Lo et al., 2009; Shaw et al., 2005). Following the publication of this study, Kulkarni and colleagues published similar results in regards to the robust expression of NiV V and W proteins due to the high frequency of NiV P gene editing observed in NiV infected cells. This study determined that the relative number of P gene mRNA transcripts encoding P

protein versus those encoding for the V and W proteins drastically decreases over the course of the first 30 hours of infection (Kulkarni et al., 2009).

A study which utilized the NiV minigenome system demonstrated that the NiV C, V, and W proteins provided in *trans* via plasmid expression inhibited NiV minigenome transcription and replication in a dose-dependent manner (Sleeman et al., 2008). Interestingly, the heterologous C and V proteins of measles virus (MeV) also had the ability to inhibit NiV minigenome replication. Conversely, NiV C, V, and W proteins were able to inhibit MeV minigenome replication, although to a lesser extent than the MeV C protein. The C protein of human parainfluenza virus 3 (hPIV3) was surprisingly also shown to inhibit replication of both NiV and MeV minigenomes. The inhibitory effect of the NiV C and V proteins on NiV minigenome replication is consistent with observations for other paramyxoviruses such as Newcastle Disease virus, MeV, hPIV2, hPIV3, and SeV (Bankamp *et al.*, 2005; Grogan & Moyer, 2001; Nishio *et al.*, 2008; Reutter *et al.*, 2001; Witko *et al.*, 2006).

The Innate Cellular Antiviral Response

In order to successfully clear a viral infection to which one has no pre-existing immunity, the innate antiviral immune response must be able to keep viral replication at levels that will not kill the host while the adaptive immune response is being primed to eventually clear the infection. There are two crucial aspects to mounting an effective innate antiviral response: 1) pathogen component recognition, and 2) the antiviral cellular response to the recognized pathogen. Various intracellular cytoplasmic and endosomal pattern recognition receptors (PRRs) such as Toll-Like receptor 3 (TLR-3), TLR 7 & 8,

Retinoic acid-inducible gene 1 (RIG-I), and melanoma differentiation-associated gene 5 (Mda-5) detect viral nucleic acids such as tri-phosphorylated RNA and single or double-stranded RNA (ssRNA, dsRNA), and in turn initiate the antiviral response (Kawai & Akira, 2008). A common and crucial aspect of the antiviral response induced by these PRRs is the production of interferons (IFNs). IFNs are soluble factors that mediate cellular antiviral responses and are classified into type I, type II, and type III IFNs. Most cells in the body produce type I IFN (IFN α/β) and type III IFN (IFN λ), while activated T lymphocytes and natural killer (NK) cells are the primary sources of type II IFN (IFNγ) (Platanias, 2005; Uze & Monneron, 2007). The transcriptional activators NF-κB, ATF2-c-Jun, interferon regulatory factor 3 (IRF-3), and IRF-7 regulate the expression of type I IFNs, with IRF-3 and IRF-7 being the primary activators of type I IFN during viral infection. While RIG-I, Mda-5, and TLR-3 induce production of type I IFN (IFN-β) via IRF-3 activation (Figure 4), TLR-7 and TLR-8 induce type I (IFN-α) production via IRF-7 activation (Kawai & Akira, 2008).

When secreted type I IFNs engage the receptor subunits IFN-α receptors 1 and 2 (IFNAR 1/2) on the cell surface, Janus tyrosine kinases (JAKs) (i.e., Jak1, Tyk2) associated with the cytoplasmic domain of these subunits phosphorylate specific tyrosine residues of the IFNARs. Signal Transducing Activators of Transcription (STAT) 1 and STAT2, which are transcription factors crucial to the IFN response, are then recruited to the phosphorylated IFNAR complex, where they are phosphorylated by JAKs at specific tyrosine and serine residues. This phosphorylation step allows STAT1 and 2 to form heterodimers that enter the nucleus, where it combines with another transcription factor, IRF-9 to form the transcriptional activator IFN-stimulated Gene Factor 3 (ISGF3).

ISGF3 binds to promoters with IFN-stimulated response element (ISRE) sequences to induce transcription of IFN-stimulated genes (ISGs) (Platanias, 2005). Some of these ISGs include IRF-7, 2'5' Oligoadenylate Synthetase (OAS), RnaseL, p56, and double-stranded RNA-induced protein kinase (PKR). These ISGs all contribute to the generation of an antiviral state in the cell (Platanias, 2005).

Virus-Host interactions of the NiV P gene products

There is extensive evidence that the paramyovirus C and V proteins can limit the induction of type I IFNs and also block IFN signaling (Lamb & Parks, 2007). Several studies utilizing eukaryotic plasmid expression systems have provided insight into possible mechanisms by which NiV C, V, and W proteins block the host antiviral response.

Initial studies of the NiV V protein indicated that it inhibits IFN signal transduction by sequestering STAT1 and STAT2 in high molecular weight complexes in the cytoplasm and by inhibiting STAT1 phosphorylation (Figure 3) (Rodriguez *et al.*, 2002). By generating truncation mutants of NiV V, this same group identified a nuclear export sequence (NES) between amino acids 174 and 192 in the N-terminus of V that enabled it to shuttle steady-state STAT1 to the cytoplasm. Attachment of this NES to a nuclear export-deficient human immunodeficiency virus Rev protein restored its subcellular location to the cytoplasm. Through using the mentioned truncation mutants of NiV V, the authors were able to map the functional domains of V responsible for binding STAT1 (amino acids 100-160). Despite finding a STAT2 binding site on V

(amino acids 230-271), it was shown that STAT2 binding to V required the presence of STAT1 (Rodriguez *et al.*, 2004).

Through using an IFN-sensitive Newcastle Disease Virus (NDV) that contained a GFP reporter gene, Park and colleagues were able to rescue replication of NDV in IFN-treated chicken embryo fibroblasts (CEFs) when they transfected plasmids expressing NiV V, W, or C proteins. The V and W proteins rescued GFP expression in a robust manner, while the level of GFP rescued by the C protein was less pronounced. It was shown that both the V and W proteins inhibited IFN signaling by an IFN-inducible promoter reporter gene assay (Park et al., 2003). This group eventually elucidated cellular localizations of V and W by immunofluorescence, showing that V localized to the cytoplasm while W was distributed exclusively to the nucleus. In addition, the STAT1 binding domain was mapped to the 50-150 amino acid region of the N-terminus shared by the P, V, and W proteins, which corresponded with the results obtained for NiV V by Rodriguez and colleagues (Rodriguez *et al.*, 2004; Shaw *et al.*, 2004). Interestingly, in spite of the shared N-terminus, it was shown that P, V, and W have differential abilities to inhibit induction of ISRE promoters, with W having the most inhibitive capacity, then V, and then P having the least capacity for inhibition (Shaw et al., 2004).

Shaw and colleagues delineated a nuclear localization signal (NLS) in the unique C-terminus of the NiV W protein, which required basic residues at amino acid positions 439, 440, and 442 (Shaw et al., 2005). The NLS was shown to interact with the nuclear importins karyopherin-α3 and karyopherin-α4. While NiV V and W could inhibit IRF-3 responsive promoter activation both by Sendai virus infection and cytoplasmic dsRNA, only W could inhibit IRF-3 activation via dsRNA stimulation of TLR-3 (Figure 4). An

interesting observation in this study was that the addition of a non-viral nuclear localization signal to the V protein resulted in the same inhibitory effect seen with W. This study also demonstrated that plasmid expression of the W protein in cells reduced levels of phosphorylated IRF-3, indicating a distinct mechanism of IFN evasion unique to W. There was a demonstrable difference in ability of the V and W proteins to inhibit the IKK-like kinases IKKε and TBK-1. Both V and W inhibited IKKε–mediated activation of ISG transcription, while only W was able to inhibit TBK-1-mediated activation of ISG transcription. The authors concluded that because W localizes to the nucleus, it could inhibit both pathways leading to IRF-3 activation, the TLR-3 pathway and the virus/dsRNA pathway (Shaw et al., 2005).

Through a serendipitous point mutation in the NiV V protein generated by PCR, Hagmaier and colleagues were able to show that a single amino acid change in residue 125 abolished STAT1 binding ability, as well as the ability to block IFN signaling. It was also shown in this study that NiV was able to block IFN signaling in cell lines from numerous mammalian species, indicating that this ability does not constrain the virus from crossing species (Hagmaier et al., 2006). In a more recent study, several glycine residues crucial for STAT-1 binding and NiV minigenome RNA replication were identified in the shared region of the P, V, and W proteins using site-directed mutagenesis (Ciancanelli et al., 2009).

It has been shown that plasmid expressed NiV V, along with many other paramyxovirus V proteins bind the helicase Mda-5 via its cysteine-rich C-terminal domain, and blocks activation of the IFN-β promoter by preventing the oligomerization of Mda-5 upon binding to dsRNA (Childs et al., 2007; Childs et al., 2009). A novel

interaction between NiV V and a host cell kinase Polo-like kinase 1 (PLK1) has recently been demonstrated (Ludlow et al., 2008). Intriguingly, the binding site for PLK1 overlaps with the STAT1 binding region. By constructing point mutants in the shared binding region, Ludlow and colleagues were able to abrogate STAT-1 binding independently of PLK1 binding to NiV V. It was shown that ablating the binding sites to either protein on the NiV P protein did not affect NiV minigenome replication, which indicates the possibility of attenuating NiV by disrupting host protein interactions via reverse genetics. The plasmid expression studies reviewed above have indeed shed significant light on the virus-host interactions of NiV, as well as the potential mechanisms of interferon antagonism by NiV V, W, and C. With the availability of a NiV reverse genetic system, the specific roles that these proteins have in pathogenesis and replication during infection can be more precisely studied (Yoneda et al., 2006). One recent study using reverse genetics determined that a NiV mutant virus with a non-functional C protein ORF drastically decreased its growth kinetics, both in IFN-β producing (HEK 293T) and non-IFN-β producing (Vero E6) cell lines. By using a STAT-1-GFP fusion protein, the study also showed that wild-type NiV infection sequestered STAT-1 in the nucleus of Vero cells, and that a STAT-1 binding mutant NiV (G121E) could not sequester STAT-1 in the same manner. Immunofluorescence assays of NiV infected cells in this study demonstrated that the phosphorylated form of STAT-1 did not localize to the nucleus despite IFN-β treatment (Ciancanelli et al., 2009). In spite of these initial studies, it remains to be seen as to whether NiV infection of physiological targets such as neurons and endothelial cells will mirror the results seen in NiV infected Vero and 293T cells.

References

Aguilar, H. C., Ataman, Z. A., Aspericueta, V., Fang, A. Q., Stroud, M., Negrete, O. A., Kammerer, R. A. & Lee, B. (2009). A novel receptor-induced activation site in the Nipah virus attachment glycoprotein (G) involved in triggering the fusion glycoprotein (F). *J Biol Chem* **284**, 1628-1635.

Aguilar, H. C., Matreyek, K. A., Choi, D. Y., Filone, C. M., Young, S. & Lee, B. (2007). Polybasic KKR motif in the cytoplasmic tail of Nipah virus fusion protein modulates membrane fusion by inside-out signaling. *J Virol* **81**, 4520-4532.

Aguilar, H. C., Matreyek, K. A., Filone, C. M., Hashimi, S. T., Levroney, E. L., Negrete, O. A., Bertolotti-Ciarlet, A., Choi, D. Y., McHardy, I., Fulcher, J. A., Su, S. V., Wolf, M. C., Kohatsu, L., Baum, L. G. & Lee, B. (2006). N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. *J Virol* **80**, 4878-4889.

Amal, N. M., Lye, M. S., Ksiazek, T. G., Kitsutani, P. D., Hanjeet, K. S., Kamaluddin, M. A., Ong, F., Devi, S., Stockton, P. C., Ghazali, O., Zainab, R. & Taha, M. A. (2000). Risk factors for Nipah virus transmission, Port Dickson, Negeri Sembilan, Malaysia: results from a hospital-based case-control study. *Southeast Asian J Trop Med Public Health* **31**, 301-306.

Appel, M. J. & Summers, B. A. (1995). Pathogenicity of morbilliviruses for terrestrial carnivores. *Vet Microbiol* **44**, 187-191.

Bankamp, B., Horikami, S. M., Thompson, P. D., Huber, M., Billeter, M. & Moyer, S. A. (1996). Domains of the measles virus N protein required for binding to P protein and self-assembly. *Virology* **216**, 272-277.

Bankamp, B., Wilson, J., Bellini, W. J. & Rota, P. A. (2005). Identification of naturally occurring amino acid variations that affect the ability of the measles virus C protein to regulate genome replication and transcription. *Virology* **336**, 120-129.

Bellini, W. J., Rota, P. A. & Anderson, L. J. (1998). Paramyxoviruses. In *Topley & Wilson's microbiology and microbial infections, 9th edition*, pp. 435-462. Edited by B. W. J. Mahy & L. Collier. London: Edward Arnold.

Bojinova, V. S., Dimova, P. S., Belopitova, L. D., Mihailov, A. S., Gatcheva, N. L., Mihneva, Z. G. & Hristova, M. T. (2004). Clinical and epidemiological characteristics of subacute sclerosing panencephalitis in Bulgaria during the past 25 years (1978-2002). *Eur J Paediatr Neurol* **8**, 89-94.

Bonaparte, M. I., Dimitrov, A. S., Bossart, K. N., Crameri, G., Mungall, B. A., Bishop, K. A., Choudhry, V., Dimitrov, D. S., Wang, L. F., Eaton, B. T. & Broder, C. C. (2005). Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. *Proc Natl Acad Sci U S A* **102**, 10652-10657.

Bossart, K. N., Wang, L. F., Eaton, B. T. & Broder, C. C. (2001). Functional expression and membrane fusion tropism of the envelope glycoproteins of Hendra virus. *Virology* **290**, 121-135.

Bowden, T. A., Aricescu, A. R., Gilbert, R. J., Grimes, J. M., Jones, E. Y. & Stuart, D. I. (2008a). Structural basis of Nipah and Hendra virus attachment to their cell-surface receptor ephrin-B2. *Nat Struct Mol Biol*.

Bowden, T. A., Crispin, M., Harvey, D. J., Aricescu, A. R., Grimes, J. M., Jones, E. Y. & Stuart, D. I. (2008b). Crystal structure and carbohydrate analysis of nipah virus attachment glycoprotein: a template for antiviral and vaccine design. *J Virol* **82**, 11628-11636.

Calain, P. & Roux, L. (1993). The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. *J Virol* **67**, 4822-4830.

Carsillo, T., Zhang, X., Vasconcelos, D., Niewiesk, S. & Oglesbee, M. (2006). A single codon in the nucleocapsid protein C terminus contributes to in vitro and in vivo fitness of Edmonston measles virus. *J Virol* **80**, 2904-2912.

CDC (1999a). Outbreak of Hendra-like virus--Malaysia and Singapore, 1998-1999. *Morbidity Mortality Wkly Rep* **48**, 265-269.

CDC (1999b). Update: outbreak of Nipah virus--Malaysia and Singapore, 1999. *Morbid Mortal Wkly Rep* **48**, 335-337.

Cevik, B., Kaesberg, J., Smallwood, S., Feller, J. A. & Moyer, S. A. (2004). Mapping the phosphoprotein binding site on Sendai virus NP protein assembled into nucleocapsids. *Virology* **325**, 216-224.

Chadha, M. S., Comer, J. A., Lowe, L., Rota, P. A., Rollin, P. E., Bellini, W. J., Ksiazek, T. G. & Mishra, A. (2006). Nipah virus-associated encephalitis outbreak, Siliguri, India. *Emerg Infect Dis* **12**, 235-240.

Chan, Y. P., Koh, C. L., Lam, S. K. & Wang, L. F. (2004). Mapping of domains responsible for nucleocapsid protein-phosphoprotein interaction of Henipaviruses. *J Gen Virol* **85**, 1675-1684.

Chattopadhyay, A., Raha, T. & Shaila, M. S. (2004). Effect of single amino acid mutations in the conserved GDNQ motif of L protein of Rinderpest virus on RNA synthesis in vitro and in vivo. *Virus Res* **99**, 139-145.

Chew, M. H., Arguin, P. M., Shay, D. K., Goh, K. T., Rollin, P. E., Shieh, W. J., Zaki, S. R., Rota, P. A., Ling, A. E., Ksiazek, T. G., Chew, S. K. & Anderson, L. J. (2000). Risk factors for Nipah virus infection among abattoir workers in Singapore. *J Infect Dis* **181**, 1760-1763.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R. & Goodbourn, S. (2007). mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* **359**, 190-200.

Childs, K. S., Andrejeva, J., Randall, R. E. & Goodbourn, S. (2009). Mechanism of mda-5 Inhibition by paramyxovirus V proteins. *J Virol* **83**, 1465-1473.

Chong, H. T., Kamarulzaman, A., Tan, C. T., Goh, K. J., Thayaparan, T., Kunjapan, S. R., Chew, N. K., Chua, K. B. & Lam, S. K. (2001). Treatment of acute Nipah encephalitis with ribavirin. *Ann Neurol* **49**, 810-813.

Chong, H. T. & Tan, C. T. (2003). Relapsed and late-onset Nipah encephalitis, a report of three cases. *Neurology Journal Southeast Asia* **8**, 109-112.

Chua, K. B., Bellini, W. J., Rota, P. A., Harcourt, B. H., Tamin, A., Lam, S. K., Ksiazek, T. G., Rollin, P. E., Zaki, S. R., Shieh, W., Goldsmith, C. S., Gubler, D. J., Roehrig, J. T., Eaton, B., Gould, A. R., Olson, J., Field, H., Daniels, P., Ling, A. E., Peters, C. J., Anderson, L. J. & Mahy, B. W. (2000). Nipah virus: a recently emergent deadly paramyxovirus. *Science* **288**, 1432-1435.

Chua, K. B., Koh, C. L., Hooi, P. S., Wee, K. F., Khong, J. H., Chua, B. H., Chan, Y. P., Lim, M. E. & Lam, S. K. (2002). Isolation of Nipah virus from Malaysian Island flying-foxes. *Microbes Infect* **4**, 145-151.

Chua, K. B., Lam, S. K., Goh, K. J., Hooi, P. S., Ksiazek, T. G., Kamarulzaman, A., Olson, J. & Tan, C. T. (2001). The presence of Nipah virus in respiratory secretions and urine of patients during an outbreak of Nipah virus encephalitis in Malaysia. *J Infect* **42**, 40-43.

Ciancanelli, M. J. & Basler, C. F. (2006). Mutation of YMYL in the Nipah virus matrix protein abrogates budding and alters subcellular localization. *J Virol* **80**, 12070-12078.

Ciancanelli, M. J., Volchkova, V. A., Shaw, M. L., Volchkov, V. E. & Basler, C. F. (2009). Nipah virus sequesters inactive STAT1 in the nucleus via a P gene-encoded mechanism. *J Virol* **83**, 7828-7841.

Corey, E. A. & Iorio, R. M. (2007). Mutations in the stalk of the measles virus hemagglutinin protein decrease fusion but do not interfere with virus-specific interaction with the homologous fusion protein. *J Virol* **81**, 9900-9910.

Corey, E. A. & Iorio, R. M. (2009). Measles virus attachment proteins with impaired ability to bind CD46 interact more efficiently with the homologous fusion protein. *Virology* **383**, 1-5.

Curran, J., Boeck, R. & Kolakofsky, D. (1991). The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. *EMBO J* **10**, 3079-3085.

Curran, J. & Kolakofsky, D. (1988a). Ribosomal initiation from an ACG codon in the Sendai virus P/C mRNA. *EMBO J* **7**, 245-251.

Curran, J. & Kolakofsky, D. (1988b). Scanning independent ribosomal initiation of the Sendai virus X protein. *EMBO J* **7**, 2869-2874.

Curran, J. & Kolakofsky, D. (1989). Scanning independent ribosomal initiation of the Sendai virus Y proteins in vitro and in vivo. *EMBO J* **8**, 521-526.

Curran, J., Marq, J. B. & Kolakofsky, D. (1995). An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. *J Virol* **69**, 849-855.

Daniels, P., Ksiazek, T. & Eaton, B. T. (2001). Laboratory diagnosis of Nipah and Hendra virus infections. *Microbes Infect* **3**, 289-295.

De, B. P., Hoffman, M. A., Choudhary, S., Huntley, C. C. & Banerjee, A. K. (2000). Role of NH(2)- and COOH-terminal domains of the P protein of human parainfluenza virus type 3 in transcription and replication. *J Virol* **74**, 5886-5895.

Devaux, P. & Cattaneo, R. (2004). Measles virus phosphoprotein gene products: conformational flexibility of the P/V protein amino-terminal domain and C protein infectivity factor function. *J Virol* **78**, 11632-11640.

Devaux, P., Hodge, G., McChesney, M. B. & Cattaneo, R. (2008). Attenuation of V- or C-defective measles viruses: infection control by the inflammatory and interferon responses of rhesus monkeys. *J Virol* **82**, 5359-5367.

Diederich, S., Dietzel, E. & Maisner, A. (2009). Nipah virus fusion protein: influence of cleavage site mutations on the cleavability by cathepsin L, trypsin and furin. *Virus Res* **145**, 300-306.

Diederich, S., Moll, M., Klenk, H. D. & Maisner, A. (2005). The nipah virus fusion protein is cleaved within the endosomal compartment. *J Biol Chem* **280**, 29899-29903.

Diederich, S., Thiel, L. & Maisner, A. (2008). Role of endocytosis and cathepsin-mediated activation in Nipah virus entry. *Virology* **375**, 391-400.

Doms, R. W., Lamb, R. A., Rose, J. K. & Helenius, A. (1993). Folding and assembly of viral membrane proteins. *Virology* **193**, 545-562.

Drexler, J. F., Corman, V. M., Gloza-Rausch, F., Seebens, A., Annan, A., Ipsen, A., Kruppa, T., Muller, M. A., Kalko, E. K., Adu-Sarkodie, Y., Oppong, S. & Drosten, C. (2009). Henipavirus RNA in African bats. *PLoS One* **4**, e6367.

Dubois, B., Lemmens, R., Laffut, W. & Van Ranst, M. (2005). Subacute sclerosing panencephalitis in the differential diagnosis of encephalitis. *Neurology* **65**, 1145-1146; author reply 1145-1146.

Durbin, A. P., Siew, J. W., Murphy, B. R. & Collins, P. L. (1997). Minimum protein requirements for transcription and RNA replication of a minigenome of human parainfluenza virus type 3 and evaluation of the rule of six. *Virology* **234**, 74-83.

Eaton, B. T., Wright, P. J., Wang, L. F., Sergeyev, O., Michalski, W. P., Bossart, K. N. & Broder, C. C. (2004). Henipaviruses: recent observations on regulation of transcription and the nature of the cell receptor. *Arch Virol Suppl*, 122-131.

Egelman, E. H., Wu, S. S., Amrein, M., Portner, A. & Murti, G. (1989). The Sendai virus nucleocapsid exists in at least four different helical states. *J Virol* **63**, 2233-2243.

Erbar, S., Diederich, S. & Maisner, A. (2008). Selective receptor expression restricts Nipah virus infection of endothelial cells. *Virol J* **5**, 142.

Errington, W. & Emmerson, P. T. (1997). Assembly of recombinant Newcastle disease virus nucleocapsid protein into nucleocapsid-like structures is inhibited by the phosphoprotein. *J Gen Virol* **78 (Pt 9)**, 2335-2339.

Freed, E. O. (2002). Viral late domains. *J Virol* **76**, 4679-4687.

Garcin, D., Itoh, M. & Kolakofsky, D. (1997). A point mutation in the Sendai virus accessory C proteins attenuates virulence for mice, but not virus growth in cell culture. *Virology* **238**, 424-431.

Garcin, D., Latorre, P. & Kolakofsky, D. (1999). Sendai virus C proteins counteract the interferon-mediated induction of an antiviral state. *J Virol* **73**, 6559-6565.

Georges-Courbot, M. C., Contamin, H., Faure, C., Loth, P., Baize, S., Leyssen, P., Neyts, J. & Deubel, V. (2006). Poly(I)-poly(C12U) but not ribavirin prevents death in a hamster model of Nipah virus infection. *Antimicrob Agents Chemother* **50**, 1768-1772.

Goh, K. J., Tan, C. T., Chew, N. K., Tan, P. S., Kamarulzaman, A., Sarji, S. A., Wong, K. T., Abdullah, B. J., Chua, K. B. & Lam, S. K. (2000). Clinical features of Nipah virus encephalitis among pig farmers in Malaysia. *N Engl J Med* **342**, 1229-1235.

Goldsmith, C. S., Whistler, T., Rollin, P. E., Ksiazek, T. G., Rota, P. A., Bellini, W. J., Daszak, P., Wong, K. T., Shieh, W. J. & Zaki, S. R. (2003). Elucidation of Nipah virus morphogenesis and replication using ultrastructural and molecular approaches. *Virus Res* **92**, 89-98.

Grogan, C. C. & Moyer, S. A. (2001). Sendai virus wild-type and mutant C proteins show a direct correlation between L polymerase binding and inhibition of viral RNA synthesis. *Virology* **288**, 96-108.

Guillaume, V., Contamin, H., Loth, P., Grosjean, I., Courbot, M. C., Deubel, V., Buckland, R. & Wild, T. F. (2006). Antibody prophylaxis and therapy against Nipah virus infection in hamsters. *J Virol* **80**, 1972-1978.

Gurley, E. S., Montgomery, J. M., Hossain, M. J., Bell, M., Azad, A. K., Islam, M. R., Molla, M. A., Carroll, D. S., Ksiazek, T. G., Rota, P. A., Lowe, L., Comer, J. A., Rollin, P., Czub, M., Grolla, A., Feldmann, H., Luby, S. P., Woodward, J. L. & Breiman, R. F. (2007a). Person-to-person transmission of Nipah virus in a Bangladeshi community. *Emerg Infect Dis* **13**, 1031-1037.

Gurley, E. S., Montgomery, J. M., Hossain, M. J., Islam, M. R., Molla, M. A., Shamsuzzaman, S. M., Akram, K., Zaman, K., Asgari, N., Comer, J. A., Azad, A. K., Rollin, P. E., Ksiazek, T. G. & Breiman, R. F. (2007b). Risk of nosocomial transmission of Nipah virus in a Bangladesh hospital. *Infect Control Hosp Epidemiol* **28**, 740-742.

Hagmaier, K., Stock, N., Goodbourn, S., Wang, L. F. & Randall, R. (2006). A single amino acid substitution in the V protein of Nipah virus alters its ability to block interferon signalling in cells from different species. *J Gen Virol* **87**, 3649-3653.

Hall, L. & Richards, G. (2000). *Flying Foxes: Fruit and Blossom Bats of Australia*. Sydney: University of New South Wales Press.

Halpin, K., Bankamp, B., Harcourt, B. H., Bellini, W. J. & Rota, P. A. (2004). Nipah virus conforms to the rule of six in a minigenome replication assay. *J Gen Virol* **85**, 701-707.

Halpin, K., Young, P. L., Field, H. E. & Mackenzie, J. S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J Gen Virol* **81**, 1927-1932.

Harcourt, B. H., Lowe, L., Tamin, A., Liu, X., Bankamp, B., Bowden, N., Rollin, P. E., Comer, J. A., Ksiazek, T. G., Hossain, M. J., Gurley, E. S., Breiman, R. F., Bellini, W. J. & Rota, P. A. (2005). Genetic characterization of Nipah virus, Bangladesh, 2004. *Emerg Infect Dis* **11**, 1594-1597.

Harcourt, B. H., Tamin, A., Halpin, K., Ksiazek, T. G., Rollin, P. E., Bellini, W. J. & Rota, P. A. (2001). Molecular characterization of the polymerase gene and genomic termini of Nipah virus. *Virology* **287**, 192-201.

Harcourt, B. H., Tamin, A., Ksiazek, T. G., Rollin, P. E., Anderson, L. J., Bellini, W. J. & Rota, P. A. (2000). Molecular characterization of Nipah virus, a newly emergent paramyxovirus. *Virology* **271**, 334-349.

Harit, A. K., Ichhpujani, R. L., Gupta, S., Gill, K. S., Lal, S., Ganguly, N. K. & Agarwal, S. P. (2006). Nipah/Hendra virus outbreak in Siliguri, West Bengal, India in 2001. *Indian J Med Res* **123**, 553-560.

Harty, R. N. & Palese, P. (1995). Measles virus phosphoprotein (P) requires the NH2- and COOH-terminal domains for interactions with the nucleoprotein (N) but only the COOH terminus for interactions with itself. *J Gen Virol* **76 (Pt 11)**, 2863-2867.

Hausmann, S., Jacques, J. P. & Kolakofsky, D. (1996). Paramyxovirus RNA editing and the requirement for hexamer genome length. *RNA* **2**, 1033-1045.

Hayman, D. T., Suu-Ire, R., Breed, A. C., McEachern, J. A., Wang, L., Wood, J. L. & Cunningham, A. A. (2008). Evidence of henipavirus infection in West African fruit bats. *PLoS ONE* **3**, e2739.

Hooper, P., Zaki, S., Daniels, P. & Middleton, D. (2001). Comparative pathology of the diseases caused by Hendra and Nipah viruses. *Microbes Infect* **3**, 315-322.

Hooper, P. T., Gould, A. R., Hyatt, A. D., Braun, M. A., Kattenbelt, J. A., Hengstberger, S. G. & Westbury, H. A. (2000). Identification and molecular characterization of Hendra virus in a horse in Queensland. *Aust Vet J* **78**, 281-282.

Horikami, S. M., Curran, J., Kolakofsky, D. & Moyer, S. A. (1992). Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *J Virol* **66**, 4901-4908.

Hossain, M. J., Gurley, E. S., Montgomery, J. M., Bell, M., Carroll, D. S., Hsu, V. P., Formenty, P., Croisier, A., Bertherat, E., Faiz, M. A., Azad, A. K., Islam, R., Molla, M. A., Ksiazek, T. G., Rota, P. A., Comer, J. A., Rollin, P. E., Luby, S. P. & Breiman, R. F. (2008). Clinical presentation of nipah virus infection in bangladesh. *Clin Infect Dis* **46**, 977-984.

Houben, K., Marion, D., Tarbouriech, N., Ruigrok, R. W. & Blanchard, L. (2007). Interaction of the C-terminal domains of sendai virus N and P proteins: comparison of polymerase-nucleocapsid interactions within the paramyxovirus family. *J Virol* **81**, 6807-6816.

Hsu, V. P., Hossain, M. J., Parashar, U. D., Ali, M. M., Ksiazek, T. G., Kuzmin, I., Niezgoda, M., Rupprecht, C., Bresee, J. & Breiman, R. F. (2004). Nipah virus encephalitis reemergence, Bangladesh. *Emerg Infect Dis* **10**, 2082-2087.

Hyatt, A. D., Zaki, S. R., Goldsmith, C. S., Wise, T. G. & Hengstberger, S. G. (2001). Ultrastructure of Hendra virus and Nipah virus within cultured cells and host animals. *Microbes Infect* **3**, 297-306.

ICDDRB (2004a). Nipah encephalitis outbreak over a wide area of Bangladesh. *Hlth Sci Bull* **2**, 7-11.

ICDDRB (2004b). Nipah encephalitis outbreak over a wide area of Bangladesh, 2004. *Hlth Sci Bull* **2**, 7-11.

ICDDRB (2004c). Person-to-person transmission of Nipah virus during outbreak in Faridpur District. *Hllth Sci Bull* **2**, 5-9.

ICDDRB (2004d). Person-to-person transmission of Nipah virus during outbreak in Faridpur District. *Health Science Bulletin* **2**, 7-11.

Irie, T., Shimazu, Y., Yoshida, T. & Sakaguchi, T. (2007). The YLDL sequence within Sendai virus M protein is critical for budding of virus-like particles and interacts with Alix/AIP1 independently of C protein. *J Virol* **81**, 2263-2273.

Jack, P. J., Boyle, D. B., Eaton, B. T. & Wang, L. F. (2005). The complete genome sequence of J virus reveals a unique genome structure in the family Paramyxoviridae. *J Virol* **79**, 10690-10700.

Jiang, Y., Liu, H., Liu, P. & Kong, X. (2008). Plasmids driven minigenome rescue system for Newcastle disease virus V4 strain. *Mol Biol Rep*.

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T. & Nagai, Y. (1997a). The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. *EMBO J* **16**, 578-587.

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T., Shioda, T. & Nagai, Y. (1997b). Importance of the cysteine-rich carboxyl-terminal half of V protein for Sendai virus pathogenesis. *J Virol* **71**, 7266-7272.

Kawai, T. & Akira, S. (2008). Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* **1143**, 1-20.

Keller, M. A., Murphy, S. K. & Parks, G. D. (2001). RNA replication from the simian virus 5 antigenomic promoter requires three sequence-dependent elements separated by sequence-independent spacer regions. *J Virol* **75**, 3993-3998.

Klenk, H. D. & Garten, W. (1994). Host cell proteases controlling virus pathogenicity. *Trends Microbiol* **2**, 39-43.

Kolakofsky, D., Pelet, T., Garcin, D., Hausmann, S., Curran, J. & Roux, L. (1998). Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *J Virol* **72**, 891-899.

Kulkarni, S., Volchkova, V., Basler, C. F., Palese, P., Volchkov, V. E. & Shaw, M. L. (2009). Nipah Virus Edits its P Gene at High Frequency to Express the V and W Proteins. *J Virol*.

Kurotani, A., Kiyotani, K., Kato, A., Shioda, T., Sakai, Y., Mizumoto, K., Yoshida, T. & Nagai, Y. (1998). Sendai virus C proteins are categorically nonessential gene products but silencing their expression severely impairs viral replication and pathogenesis. *Genes Cells* **3**, 111-124.

Lamb, R. A. & Parks, G. D. (2007). Paramyxoviridae: The viruses and Their Replication. In *Fields Virology*, 5th edn, pp. 1449-1496. Edited by H. P. Knipe DM. Philadelphia: Lippincott Williams & Wilkins.

Latorre, P., Kolakofsky, D. & Curran, J. (1998). Sendai virus Y proteins are initiated by a ribosomal shunt. *Mol Cell Biol* **18**, 5021-5031.

Lee, J. K., Prussia, A., Paal, T., White, L. K., Snyder, J. P. & Plemper, R. K. (2008). Functional interaction between paramyxovirus fusion and attachment proteins. *J Biol Chem* **283**, 16561-16572.

Levroney, E. L., Aguilar, H. C., Fulcher, J. A., Kohatsu, L., Pace, K. E., Pang, M., Gurney, K. B., Baum, L. G. & Lee, B. (2005). Novel innate immune functions for galectin-1: galectin-1 inhibits cell fusion by Nipah virus envelope glycoproteins and augments dendritic cell secretion of proinflammatory cytokines. *J Immunol* **175**, 413-420.

Li, Y., Wang, J., Hickey, A. C., Zhang, Y., Wu, Y., Zhang, H., Yuan, J., Han, Z., McEachern, J., Broder, C. C., Wang, L. F. & Shi, Z. (2008). Antibodies to Nipah or Nipah-like viruses in bats, China. *Emerg Infect Dis* **14**, 1974-1976.

Li, Z., Yu, M., Zhang, H., Magoffin, D. E., Jack, P. J., Hyatt, A., Wang, H. Y. & Wang, L. F. (2006). Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-stranded RNA viruses. *Virology* **346**, 219-228.

Liston, P., Batal, R., DiFlumeri, C. & Briedis, D. J. (1997). Protein interaction domains of the measles virus nucleocapsid protein (NP). *Arch Virol* **142**, 305-321.

Lo, M. K., Harcourt, B. H., Mungall, B. A., Tamin, A., Peeples, M. E., Bellini, W. J. & Rota, P. A. (2009). Determination of the henipavirus phosphoprotein gene mRNA editing frequencies and detection of the C, V and W proteins of Nipah virus in virus-infected cells. *J Gen Virol* **90**, 398-404.

Luby, S. P., Rahman, M., Hossain, M. J., Blum, L. S., Husain, M. M., Gurley, E., Khan, R., Ahmed, B. N., Rahman, S., Nahar, N., Kenah, E., Comer, J. A. & Ksiazek, T. G. (2006). Foodborne transmission of Nipah virus, Bangladesh. *Emerg Infect Dis* **12**, 1888-1894.

Ludlow, L. E., Lo, M. K., Rodriguez, J. J., Rota, P. A. & Horvath, C. M. (2008). Henipavirus V protein association with Polo-like kinase reveals functional overlap with STAT1 binding and interferon evasion. *J Virol* **82**, 6259-6271.

Magoffin, D. E., Halpin, K., Rota, P. A. & Wang, L. F. (2007a). Effects of single amino acid substitutions at the E residue in the conserved GDNE motif of the Nipah virus polymerase (L) protein. *Arch Virol* **152**, 827-832.

Magoffin, D. E., Mackenzie, J. S. & Wang, L. F. (2007b). Genetic analysis of J-virus and Beilong virus using minireplicons. *Virology* **364**, 103-111.

Mayo, M. A. (2002). A summary of taxonomic changes recently approved by ICTV. *Arch Virol* **147**, 1655-1663.

McEachern, J. A., Bingham, J., Crameri, G., Green, D. J., Hancock, T. J., Middleton, D., Feng, Y. R., Broder, C. C., Wang, L. F. & Bossart, K. N. (2008). A recombinant subunit vaccine formulation protects against lethal Nipah virus challenge in cats. *Vaccine* **26**, 3842-3852.

McGinnes, L., Sergel, T., Reitter, J. & Morrison, T. (2001). Carbohydrate modifications of the NDV fusion protein heptad repeat domains influence maturation and fusion activity. *Virology* **283**, 332-342.

Mehdi, H., Ono, E. & Gupta, K. C. (1990). Initiation of translation at CUG, GUG, and ACG codons in mammalian cells. *Gene* **91**, 173-178.

Melanson, V. R. & Iorio, R. M. (2004). Amino acid substitutions in the F-specific domain in the stalk of the newcastle disease virus HN protein modulate fusion and interfere with its interaction with the F protein. *J Virol* **78**, 13053-13061.

Melanson, V. R. & Iorio, R. M. (2006). Addition of N-glycans in the stalk of the Newcastle disease virus HN protein blocks its interaction with the F protein and prevents fusion. *J Virol* **80**, 623-633.

Michalski, W. P., Crameri, G., Wang, L., Shiell, B. J. & Eaton, B. (2000). The cleavage activation and sites of glycosylation in the fusion protein of Hendra virus. *Virus Res* **69**, 83-93.

Middleton, D. J., Morrissy, C. J., van der Heide, B. M., Russell, G. M., Braun, M. A., Westbury, H. A., Halpin, K. & Daniels, P. W. (2007). Experimental Nipah virus infection in pteropid bats (Pteropus poliocephalus). *J Comp Pathol* **136**, 266-272.

Middleton, D. J., Westbury, H. A., Morrissy, C. J., van der Heide, B. M., Russell, G. M., Braun, M. A. & Hyatt, A. D. (2002). Experimental Nipah virus infection in pigs and cats. *J Comp Pathol* **126**, 124-136.

Mohd, N. M. N., Gan, C. H. & Ong, B. L. (2000). Nipah virus infection of pigs in peninsular Malaysia. *Rev Sci Tech Off Int Epiz* **19**, 160-165.

Moll, M., Diederich, S., Klenk, H. D., Czub, M. & Maisner, A. (2004a). Ubiquitous activation of the Nipah virus fusion protein does not require a basic amino acid at the cleavage site. *J Virol* **78**, 9705-9712.

Moll, M., Kaufmann, A. & Maisner, A. (2004b). Influence of N-glycans on processing and biological activity of the nipah virus fusion protein. *J Virol* **78**, 7274-7278.

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A., Gleeson, L., Westbury, H., Hiley, L., Selvey, L., Rodwell, B. & et al. (1995). A morbillivirus that caused fatal disease in horses and humans. *Science* **268**, 94-97.

Negrete, O. A., Chu, D., Aguilar, H. C. & Lee, B. (2007). Single amino acid changes in the Nipah and Hendra virus attachment glycoproteins distinguish ephrinB2 from ephrinB3 usage. *J Virol* **81**, 10804-10814.

Negrete, O. A., Levroney, E. L., Aguilar, H. C., Bertolotti-Ciarlet, A., Nazarian, R., Tajyar, S. & Lee, B. (2005). EphrinB2 is the entry receptor for Nipah virus, an emergent deadly paramyxovirus. *Nature* **436**, 401-405.

Negrete, O. A., Wolf, M. C., Aguilar, H. C., Enterlein, S., Wang, W., Muhlberger, E., Su, S. V., Bertolotti-Ciarlet, A., Flick, R. & Lee, B. (2006). Two key residues in ephrinB3 are critical for its use as an alternative receptor for Nipah virus. *PLoS Pathog* **2**, e7.

Nishio, M., Ohtsuka, J., Tsurudome, M., Nosaka, T. & Kolakofsky, D. (2008). Human parainfluenza virus type 2 V protein inhibits genome replication by binding to the L protein: possible role in promoting viral fitness. *J Virol* **82**, 6130-6138.

O'Sullivan, J. D., Allworth, A. M., Paterson, D. L., Snow, T. M., Boots, R., Gleeson, L. J., Gould, A. R., Hyatt, A. D. & Bradfield, J. (1997). Fatal encephalitis due to novel paramyxovirus transmitted from horses. *Lancet* **349**, 93-95.

Ong, S. T., Yusoff, K., Kho, C. L., Abdullah, J. O. & Tan, W. S. (2009). Mutagenesis of the nucleocapsid protein of Nipah virus involved in capsid assembly. *J Gen Virol* **90**, 392-397.

Ozturk, A., Gurses, C., Baykan, B., Gokyigit, A. & Eraksoy, M. (2002). Subacute sclerosing panencephalitis: clinical and magnetic resonance imaging evaluation of 36 patients. *J Child Neurol* **17**, 25-29.

Pager, C. T., Craft, W. W., Jr., Patch, J. & Dutch, R. E. (2006). A mature and fusogenic form of the Nipah virus fusion protein requires proteolytic processing by cathepsin L. *Virology* **346**, 251-257.

Pallister, J., Middleton, D., Crameri, G., Yamada, M., Klein, R., Hancock, T. J., Foord, A., Shiell, B., Michalski, W., Broder, C. C. & Wang, L. F. (2009). Chloroquine administration does not prevent Nipah virus infection and disease in ferrets. *J Virol*.

Palmer, A. & Klein, R. (2003). Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. *Genes Dev* **17**, 1429-1450.

Parashar, U. D., Sunn, L. M., Ong, F., Mounts, A. W., Arif, M. T., Ksiazek, T. G., Kamaluddin, M. A., Mustafa, A. N., Kaur, H., Ding, L. M., Othman, G., Radzi, H. M., Kitsutani, P. T., Stockton, P. C., Arokiasamy, J., Gary, H. E., Jr. & Anderson, L. J. (2000). Case-control study of risk factors for human infection with a new zoonotic paramyxovirus, Nipah virus, during a 1998-1999 outbreak of severe encephalitis in Malaysia. *J Infect Dis* **181**, 1755-1759.

Park, M. S., Shaw, M. L., Munoz-Jordan, J., Cros, J. F., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A. & Basler, C. F. (2003). Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol* **77**, 1501-1511.

Patch, J. R., Crameri, G., Wang, L. F., Eaton, B. T. & Broder, C. C. (2007). Quantitative analysis of Nipah virus proteins released as virus-like particles reveals central role for the matrix protein. *Virol J* **4**, 1.

Patch, J. R., Han, Z., McCarthy, S. E., Yan, L., Wang, L. F., Harty, R. N. & Broder, C. C. (2008). The YPLGVG sequence of the Nipah virus matrix protein is required for budding. *Virol J* **5**, 137.

Paton, N. I., Leo, Y. S., Zaki, S. R., Auchus, A. P., Lee, K. E., Ling, A. E., Chew, S. K., Ang, B., Rollin, P. E., Umapathi, T., Sng, I., Lee, C. C., Lim, E. & Ksiazek, T. G. (1999). Outbreak of Nipah-virus infection among abattoir workers in Singapore. *Lancet* **354**, 1253-1256.

Patterson, J. B., Thomas, D., Lewicki, H., Billeter, M. A. & Oldstone, M. B. (2000). V and C proteins of measles virus function as virulence factors in vivo. *Virology* **267**, 80-89.

Perez, M., Sanchez, A., Cubitt, B., Rosario, D. & de la Torre, J. C. (2003). A reverse genetics system for Borna disease virus. *J Gen Virol* **84**, 3099-3104.

Pernet, O., Pohl, C., Ainouze, M., Kweder, H. & Buckland, R. (2009). Nipah virus entry can occur by macropinocytosis. *Virology*.

Platanias, L. C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-386.

Poch, O., Sauvaget, I., Delarue, M. & Tordo, N. (1989). Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *EMBO J* **8**, 3867-3874.

Poliakov, A., Cotrina, M. & Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev Cell* **7**, 465-480.

PROMED-MAIL (2007). Nipah virus, fatal - India (West Bengal).

PROMED-MAIL (2008). Nipah virus, fatal - Bangladesh.

Pulliam, J. R., Field, H. E. & Olival, K. J. (2005). Nipah virus strain variation. *Emerg Infect Dis* **11**, 1978-1979; author reply 1979.

Quddus, R., Alam, S., Majumdar, M. A., Anwar, S., Khan, M. R., Zahid Mahmud Khan, A., Arif, S. M., Alam, R., Siddiqui, F. M., Tan, C. T. & Faiz, M. A. (2004). A report of 4 patients with Nipah encephalitis from Rajbari district, Bangladesh in the January 2004 outbreak. *Neurology Asia* **9**, 33-37.

Raha, T., Chattopadhyay, A. & Shaila, M. S. (2004). Development of a reconstitution system for Rinderpest virus RNA synthesis in vitro. *Virus Res* **99**, 131-138.

Reutter, G. L., Cortese-Grogan, C., Wilson, J. & Moyer, S. A. (2001). Mutations in the measles virus C protein that up regulate viral RNA synthesis. *Virology* **285**, 100-109.

Reynes, J. M., Counor, D., Ong, S., Faure, C., Seng, V., Molia, S., Walston, J., Georges-Courbot, M. C., Deubel, V. & Sarthou, J. L. (2005). Nipah virus in Lyle's flying foxes, Cambodia. *Emerg Infect Dis* **11**, 1042-1047.

Roberts, P. C., Garten, W. & Klenk, H. D. (1993). Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. *J Virol* **67**, 3048-3060.

Rodriguez, J. J., Cruz, C. D. & Horvath, C. M. (2004). Identification of the nuclear export signal and STAT-binding domains of the Nipah virus V protein reveals mechanisms underlying interferon evasion. *J Virol* **78**, 5358-5367.

Rodriguez, J. J., Parisien, J. P. & Horvath, C. M. (2002). Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. *J Virol* **76**, 11476-11483.

Roelke-Parker, M. E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S. J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H. & et al. (1996). A canine distemper virus epidemic in Serengeti lions (Panthera leo). *Nature* **379**, 441-445.

Rosario, D., Perez, M. & de la Torre, J. C. (2005). Functional characterization of the genomic promoter of borna disease virus (BDV): implications of 3'-terminal sequence heterogeneity for BDV persistence. *J Virol* **79**, 6544-6550.

Sanderson, C. M., Wu, H. H. & Nayak, D. P. (1994). Sendai virus M protein binds independently to either the F or the HN glycoprotein in vivo. *J Virol* **68**, 69-76.

Schmitt, A. P., Leser, G. P., Morita, E., Sundquist, W. I. & Lamb, R. A. (2005). Evidence for a new viral late-domain core sequence, FPIV, necessary for budding of a paramyxovirus. *J Virol* **79**, 2988-2997.

Schnell, M. J. & Conzelmann, K. K. (1995). Polymerase activity of in vitro mutated rabies virus L protein. *Virology* **214**, 522-530.

Segawa, H., Yamashita, T., Kawakita, M. & Taira, H. (2000). Functional analysis of the individual oligosaccharide chains of sendai virus fusion protein. *J Biochem* **128**, 65-72.

Sejvar, J. J., Hossain, J., Saha, S. K., Gurley, E. S., Banu, S., Hamadani, J. D., Faiz, M. A., Siddiqui, F. M., Mohammad, Q. D., Mollah, A. H., Uddin, R., Alam, R., Rahman, R., Tan, C. T., Bellini, W., Rota, P., Breiman, R. F. & Luby, S. P. (2007). Long-term neurological and functional outcome in Nipah virus infection. *Ann Neurol* **62**, 235-242.

Selvey, L. A., Wells, R. M., McCormack, J. G., Ansford, A. J., Murray, K., Rogers, R. J., Lavercombe, P. S., Selleck, P. & Sheridan, J. W. (1995). Infection of humans and horses by a newly described morbillivirus. *Med J Aust* **162**, 642-645.

Sendow, I., Field, H. E., Curran, J., Darminto, Morrissy, C., Meehan, G., Buick, T. & Daniels, P. (2006). Henipavirus in Pteropus vampyrus Bats, Indonesia. *Emerg Infect Dis* **12**, 711-712.

Sener, R. N. (2004). Subacute sclerosing panencephalitis findings at MR imaging, diffusion MR imaging, and proton MR spectroscopy. *AJNR Am J Neuroradiol* **25**, 892-894.

Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P. & Basler, C. F. (2005). Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. *J Virol* **79**, 6078-6088.

Shaw, M. L., Garcia-Sastre, A., Palese, P. & Basler, C. F. (2004). Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. *J Virol* **78**, 5633-5641.

Shiell, B. J., Gardner, D. R., Crameri, G., Eaton, B. T. & Michalski, W. P. (2003). Sites of phosphorylation of P and V proteins from Hendra and Nipah viruses: newly emerged members of Paramyxoviridae. *Virus Res* **92**, 55-65.

Sidhu, M. S., Chan, J., Kaelin, K., Spielhofer, P., Radecke, F., Schneider, H., Masurekar, M., Dowling, P. C., Billeter, M. A. & Udem, S. A. (1995). Rescue of synthetic measles virus minireplicons: measles genomic termini direct efficient expression and propagation of a reporter gene. *Virology* **208**, 800-807.

Sleeman, K., Bankamp, B., Hummel, K. B., Lo, M. K., Bellini, W. J. & Rota, P. A. (2008). The C, V and W proteins of Nipah virus inhibit minigenome replication. *J Gen Virol* **89**, 1300-1308.

Stec, D. S., Hill, M. G., 3rd & Collins, P. L. (1991). Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. *Virology* **183**, 273-287.

Tamin, A., Harcourt, B. H., Ksiazek, T. G., Rollin, P. E., Bellini, W. J. & Rota, P. A. (2002). Functional properties of the fusion and attachment glycoproteins of Nipah virus. *Virology* **296**, 190-200.

Tamura, T., Yamashita, T., Segawa, H. & Taira, H. (2002). N-linked oligosaccharide chains of Sendai virus fusion protein determine the interaction with endoplasmic reticulum molecular chaperones. *FEBS Lett* **513**, 153-158.

Tan, C. T., Goh, K. J., Wong, K. T., Sarji, S. A., Chua, K. B., Chew, N. K., Murugasu, P., Loh, Y. L., Chong, H. T., Tan, K. S., Thayaparan, T., Kumar, S. & Jusoh, M. R. (2002). Relapsed and late-onset Nipah encephalitis. *Ann Neurol* **51**, 703-708.

Tan, W. S., Ong, S. T., Eshaghi, M., Foo, S. S. & Yusoff, K. (2004). Solubility, immunogenicity and physical properties of the nucleocapsid protein of Nipah virus produced in Escherichia coli. *J Med Virol* **73**, 105-112.

Tapparel, C., Maurice, D. & Roux, L. (1998). The activity of Sendai virus genomic and antigenomic promoters requires a second element past the leader template regions: a motif (GNNNNN)3 is essential for replication. *J Virol* **72**, 3117-3128.

Tashiro, M., McQueen, N. L., Seto, J. T., Klenk, H. D. & Rott, R. (1996). Involvement of the mutated M protein in altered budding polarity of a pantropic mutant, F1-R, of Sendai virus. *J Virol* **70**, 5990-5997.

Tashiro, M., Seto, J. T., Klenk, H. D. & Rott, R. (1993). Possible involvement of microtubule disruption in bipolar budding of a Sendai virus mutant, F1-R, in epithelial MDCK cells. *J Virol* **67**, 5902-5910.

Thiel, L., Diederich, S., Erbar, S., Pfaff, D., Augustin, H. G. & Maisner, A. (2008). Ephrin-B2 expression critically influences Nipah virus infection independent of its cytoplasmic tail. *Virol J* **5**, 163.

Tober, C., Seufert, M., Schneider, H., Billeter, M. A., Johnston, I. C., Niewiesk, S., ter Meulen, V. & Schneider-Schaulies, S. (1998). Expression of measles virus V protein is associated with pathogenicity and control of viral RNA synthesis. *J Virol* **72**, 8124-8132.

Tong, S., Li, M., Vincent, A., Compans, R. W., Fritsch, E., Beier, R., Klenk, C., Ohuchi, M. & Klenk, H. D. (2002). Regulation of fusion activity by the cytoplasmic domain of a paramyxovirus F protein. *Virology* **301**, 322-333.

Torres-Velez, F. J., Shieh, W. J., Rollin, P. E., Morken, T., Brown, C., Ksiazek, T. G. & Zaki, S. R. (2008). Histopathologic and immunohistochemical characterization of Nipah virus infection in the guinea pig. *Vet Pathol* **45**, 576-585.

Toth, A. M., Devaux, P., Cattaneo, R. & Samuel, C. E. (2009). Protein kinase PKR mediates the apoptosis induction and growth restriction phenotypes of C protein-deficient measles virus. *J Virol* **83**, 961-968.

Uze, G. & Monneron, D. (2007). IL-28 and IL-29: newcomers to the interferon family. *Biochimie* **89**, 729-734.

Valsamakis, A., Schneider, H., Auwaerter, P. G., Kaneshima, H., Billeter, M. A. & Griffin, D. E. (1998). Recombinant measles viruses with mutations in the C, V, or F gene have altered growth phenotypes in vivo. *J Virol* **72**, 7754-7761.

Vogt, C., Eickmann, M., Diederich, S., Moll, M. & Maisner, A. (2005). Endocytosis of the Nipah virus glycoproteins. *J Virol* **79**, 3865-3872.

von Messling, V., Svitek, N. & Cattaneo, R. (2006). Receptor (SLAM [CD150]) recognition and the V protein sustain swift lymphocyte-based invasion of mucosal tissue and lymphatic organs by a morbillivirus. *J Virol* **80**, 6084-6092.

Wacharapluesadee, S., Lumlertdacha, B., Boongird, K., Wanghongsa, S., Chanhome, L., Rollin, P., Stockton, P., Rupprecht, C. E., Ksiazek, T. G. & Hemachudha, T. (2005). Bat Nipah virus, Thailand. *Emerg Infect Dis* **11**, 1949-1951.

Walpita, P. & Peters, C. J. (2007). Cis-acting elements in the antigenomic promoter of Nipah virus. *J Gen Virol* **88**, 2542-2551.

Wang, L., Harcourt, B. H., Yu, M., Tamin, A., Rota, P. A., Bellini, W. J. & Eaton, B. T. (2001). Molecular biology of Hendra and Nipah viruses. *Microbes Infect* **3**, 279-287.

Wang, L. F., Michalski, W. P., Yu, M., Pritchard, L. I., Crameri, G., Shiell, B. & Eaton, B. T. (1998). A novel P/V/C gene in a new member of the Paramyxoviridae family, which causes lethal infection in humans, horses, and other animals. *Journal of Virology* **72**, 1482-1490.

Wang, L. F., Yu, M., Hansson, E., Pritchard, L. I., Shiell, B., Michalski, W. P. & Eaton, B. T. (2000a). The exceptionally large genome of hendra virus: support for creation of a new genus within the family paramyxoviridae. *Journal of Virology* **74**, 9972-9979.

Wang, L. F., Yu, M., Hansson, E., Pritchard, L. I., Shiell, B., Michalski, W. P. & Eaton, B. T. (2000b). The exceptionally large genome of Hendra virus: support for creation of a new genus within the family Paramyxoviridae. *J Virol* **74**, 9972-9979.

Waning, D. L., Russell, C. J., Jardetzky, T. S. & Lamb, R. A. (2004). Activation of a paramyxovirus fusion protein is modulated by inside-out signaling from the cytoplasmic tail. *Proc Natl Acad Sci U S A* **101**, 9217-9222.

Weingartl, H., Czub, S., Copps, J., Berhane, Y., Middleton, D., Marszal, P., Gren, J., Smith, G., Ganske, S., Manning, L. & Czub, M. (2005). Invasion of the central nervous system in a porcine host by nipah virus. *J Virol* **79**, 7528-7534.

WHO (2004). Nipah virus outbreak(s) in Bangladesh, January-April 2004. *Wkly Epidemiol Rec* **17**, 168-171.

Williamson, M. M., Hooper, P. T., Selleck, P. W., Gleeson, L. J., Daniels, P. W., Westbury, H. A. & Murray, P. K. (1998). Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. *Aust Vet J* **76**, 813-818.

Williamson, M. M., Hooper, P. T., Selleck, P. W., Westbury, H. A. & Slocombe, R. F. (2000). Experimental hendra virus infectionin pregnant guinea-pigs and fruit Bats (Pteropus poliocephalus). *J Comp Pathol* **122**, 201-207.

Witko, S. E., Kotash, C., Sidhu, M. S., Udem, S. A. & Parks, C. L. (2006). Inhibition of measles virus minireplicon-encoded reporter gene expression by V protein. *Virology* **348**, 107-119.

Wong, K. T., Shieh, W. J., Kumar, S., Norain, K., Abdullah, W., Guarner, J., Goldsmith, C. S., Chua, K. B., Lam, S. K., Tan, C. T., Goh, K. J., Chong, H. T., Jusoh, R., Rollin, P. E., Ksiazek, T. G. & Zaki, S. R. (2002). Nipah virus infection: pathology and pathogenesis of an emerging paramyxoviral zoonosis. *Am J Pathol* **161**, 2153-2167.

Xu, K., Rajashankar, K. R., Chan, Y. P., Himanen, J. P., Broder, C. C. & Nikolov, D. B. (2008). Host cell recognition by the henipaviruses: crystal structures of the Nipah G attachment glycoprotein and its complex with ephrin-B3. *Proc Natl Acad Sci U S A* **105**, 9953-9958.

Yob, J. M., Field, H., Rashdi, A. M., Morrissy, C., van der Heide, B., Rota, P., bin Adzhar, A., White, J., Daniels, P., Jamaluddin, A. & Ksiazek, T. (2001). Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. *Emerg Infect Dis* **7**, 439-441.

Yoneda, M., Guillaume, V., Ikeda, F., Sakuma, Y., Sato, H., Wild, T. F. & Kai, C. (2006). Establishment of a Nipah virus rescue system. *Proc Natl Acad Sci U S A* **103**, 16508-16513.

Yu, M., Hansson, E., Langedijk, J. P., Eaton, B. T. & Wang, L. F. (1998a). The attachment protein of Hendra virus has high structural similarity but limited primary sequence homology compared with viruses in the genus Paramyxovirus. *Virology* **251**, 227-233.

Yu, M., Hansson, E., Shiell, B., Michalski, W., Eaton, B. T. & Wang, L. F. (1998b). Sequence analysis of the Hendra virus nucleoprotein gene: comparison with other members of the subfamily Paramyxovirinae. *Journal of General Virology* **79**, 1775-1780.

Zimmer, G., Trotz, I. & Herrler, G. (2001). N-glycans of F protein differentially affect fusion activity of human respiratory syncytial virus. *J Virol* **75**, 4744-4751.

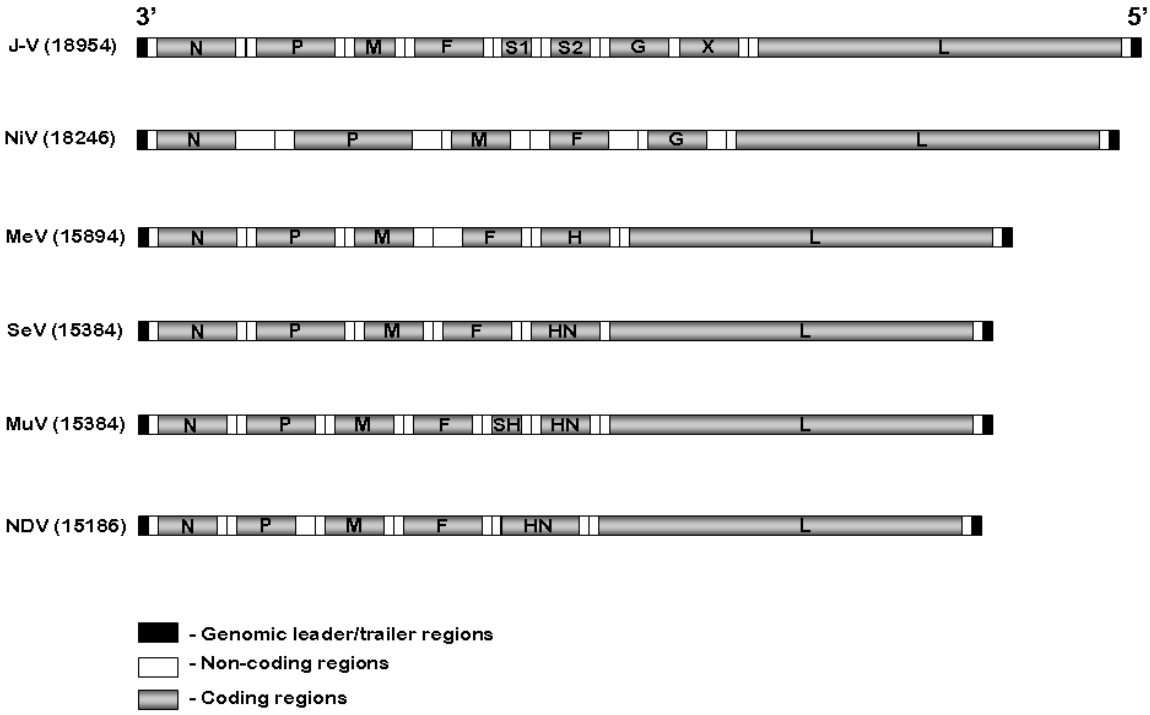


Figure 1. Comparative genome lengths among viruses in the family *Paramyxovirinae*. NiV has an exceptionally large genome compared to most paramyxoviruses by virtue of its longer non-coding regions and larger P gene (genome lengths in parentheses, see text). The solid black segments indicate leader and trailer sequences. The clear segments indicate non-coding regions, and the shaded areas indicate the respective NiV gene coding regions. J-V- J-paramyxovirus; MeV- Measles virus; SeV- Sendai virus; MuV- Mumps virus; NDV- Newcastle Disease virus.

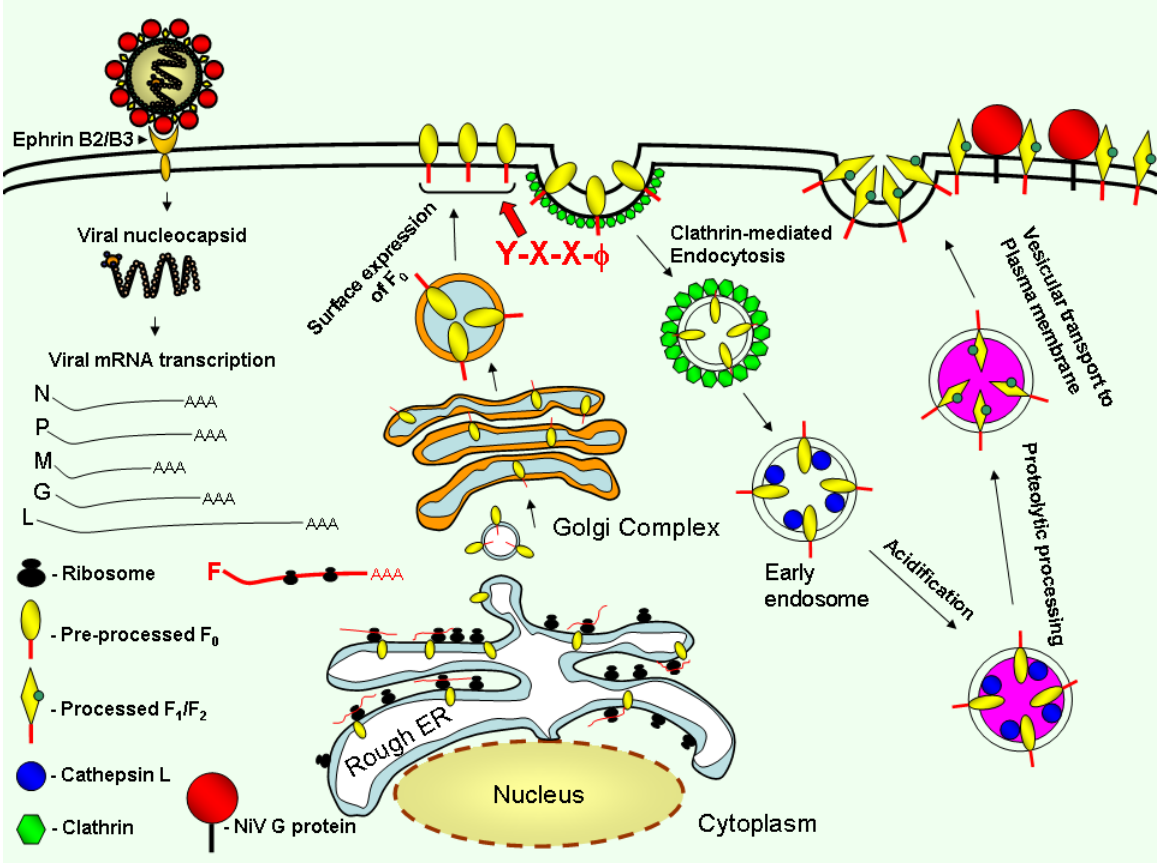


Figure 2. Proteolytic processing of the NiV F protein. The NiV F gene mRNA is translated by ribosomes into F protein, which is post-translationally modified as it progresses through the cell secretory system. Pre-processed F0 is transported to the plasma membrane, where it is endocytosed by a clathrin-dependent mechanism via its Y-X-X-ϕ motif in its C-terminus (see text). The F0 eventually reaches the early endosome, where upon its acidification, the cysteine protease cathepsin L cleaves F0 into the F_1-F_2 activated form of F. The processed F is trafficked to the plasma membrane where it can mediate cell-to-cell fusion with the NiV G protein.

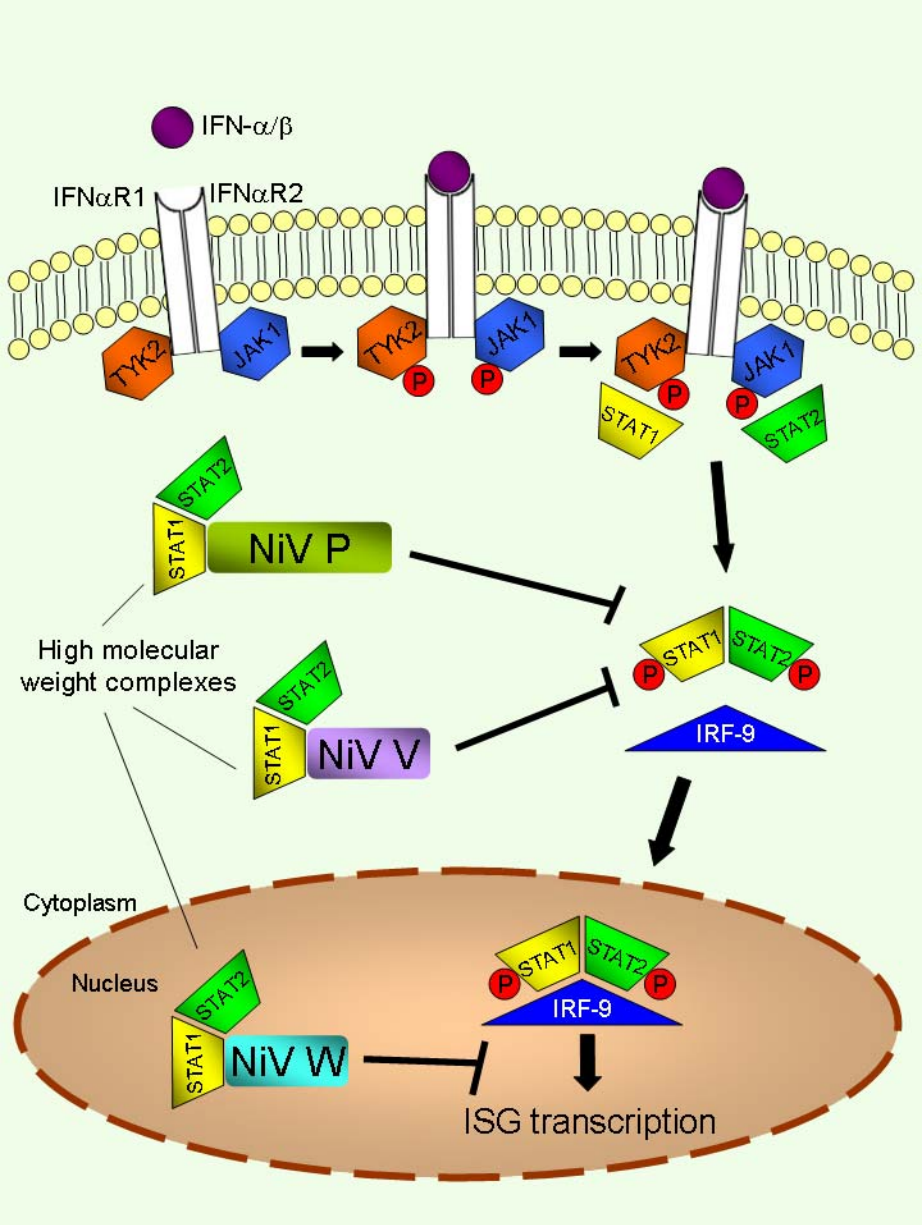


Figure 3. NiV P gene products have the ability to block IFN signaling by binding STAT-1. By means of their shared identical N-termini, the NiV P, V, and W proteins are able to prevent STAT-1 phosphorylation by Janus kinases Tyk2 and Jak1, thus blocking the formation of the ISGF3 transcription factor, which activates transcription of interferon stimulated genes (ISGs). (See text)

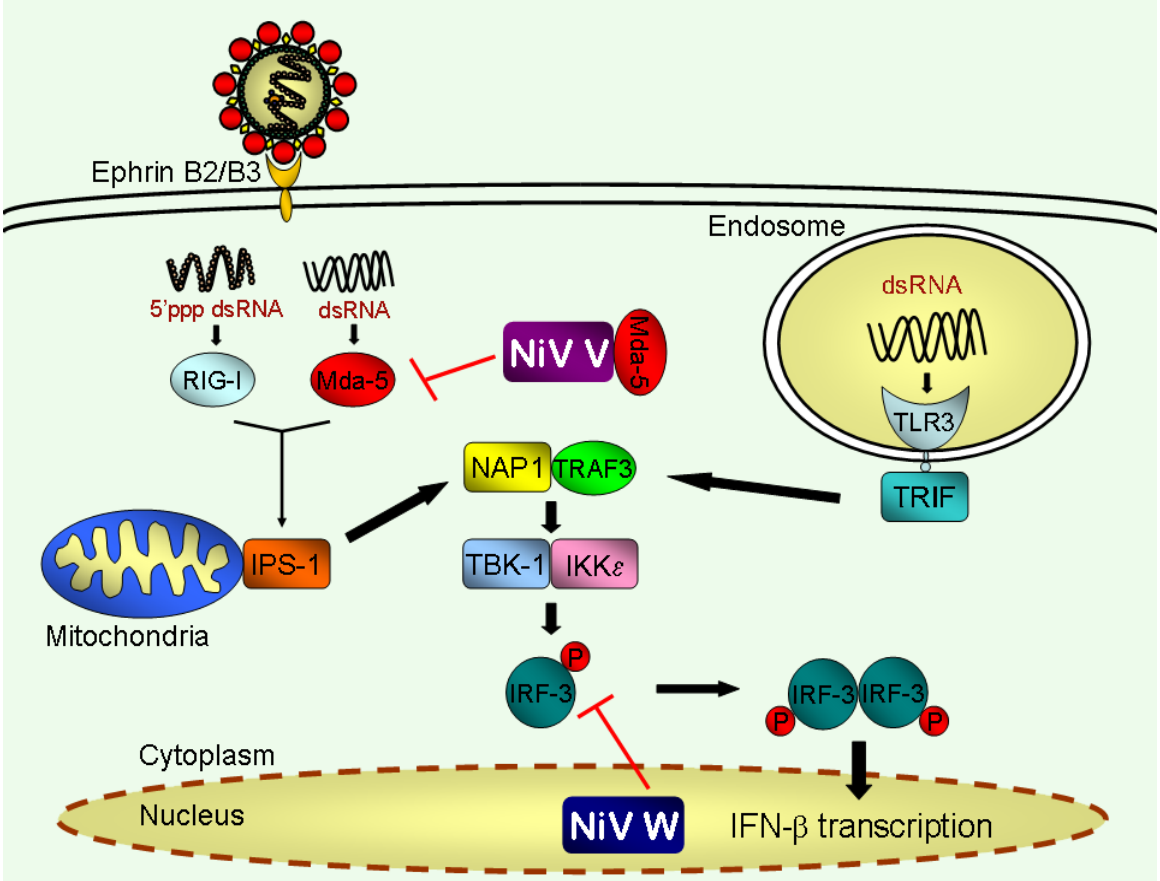


Figure 4. The interaction of NiV V and W proteins with cellular components involved in IFN induction by pattern recognition receptors (PRRs). When TLR-3 recognizes dsRNA, a molecule called TIR domain-containing adaptor inducing IFN-ß (TRIF) associates with a kinase complex of non-canonical IκB kinases (IKKs) TBK1 and IKKε that phosphorylates IRF-3. Phosphorylated IRF-3 translocates into the nucleus to activate transcription of IFN-β. RIG-I and Mda5 are two cytoplasmic PRRs involved in the induction of type I IFN. They are both DexD/H box RNA helicases with caspase recruitment domain (CARD)-like domains. These PRRs signal through a mitochondrial adaptor molecule called Interferon Promoter Stimulator-1 (IPS-1), which in turn leads to activation of TBK1 and IKKε, resulting in IRF-3 activation. Plasmid expression of NiV V and W proteins has been demonstrated to block IRF-3 activation (see text).

Chapter 2

Determination of Henipavirus P gene mRNA Editing Frequencies and Detection of the C, V, and W Proteins of Nipah Virus in Virus-Infected Cells

Michael K. Lo[1,2,4,5], Brian H. Harcourt[1], Bruce A. Mungall[3], Azaibi Tamin[1], Mark E. Peeples[4,5], William J. Bellini[1], and Paul A. Rota[1,*]

[1]Measles, Mumps, Rubella and Herpesviruses Laboratory Branch, 1600 Clifton Road, MS-C-22, Atlanta, GA 30333.

[2]Emory University, Laney Graduate School, Graduate Division of Biological and Biomedical Sciences, Immunology and Molecular Pathogenesis Program, 1462 Clifton Road Suite 314, Atlanta, GA 30322.

[3]Commonwealth Scientific Industrial Research Organization, Australian Animal Health Laboratory, 5 Portarlington Road, East Geelong, Victoria, Australia.

[4]The Research Institute at Nationwide Children's Hospital, Center for Vaccines and Immunity, 700 Children's Drive, Columbus, OH, 43205. [5]The Ohio State University, College of Medicine, Department of Pediatrics, Columbus, OH, 43205.

Contribution of other authors to this body of work: The northern blot was performed by A.T. The peptide conjugation and solubilization was performed by B.H.H. The TA cloning was performed by P.A.R. The immunofluorescence assays were performed by B.A.M.

The contents of this chapter have been published in the Journal of General Virology

Abstract

The henipaviruses, Nipah virus (NiV) and Hendra virus (HeV), are highly pathogenic zoonotic paramyxoviruses. Like many other paramyxoviruses, henipaviruses employ a process of co-transcriptional mRNA editing during transcription of the phosphoprotein (P) gene to generate additional mRNAs encoding the V and W proteins. The C protein is translated from the P mRNA, but in an alternate reading frame. Sequence analysis of multiple, cloned mRNAs showed that the mRNA editing frequencies of the P genes of the henipaviruses are higher than those reported for other paramyxoviruses. Antisera to synthetic peptides from the P, V, W, and C proteins of NiV were generated to study their expression in infected cells. All proteins were detected in both infected cells and in purified virions. In infected cells, the W protein was detected in the nucleus while P, V, and C were found in the cytoplasm. The W protein was shown to co-immunoprecipitate with karyopherin $\alpha 3$.

Introduction

Nipah virus (NiV) and Hendra virus (HeV) are paramyxoviruses in the genus *Henipavirus* of the subfamily *Paramyxovirinae* within the family *Paramyxoviridae*. HeV causes a febrile respiratory illness in humans and animals, and was responsible for the deaths of two humans out of three infections and 17 horses in three separate incidents in Australia between 1994 and 1999, and more recently in 2004, 2006, and 2007 (Hanna *et al.*, 2006; Hooper & Williamson, 2000; Murray *et al.*, 1995a; Murray *et al.*, 1995b; Promed-mail, 2006; 2007a; b). The first known human infections with NiV were detected during an outbreak of severe febrile encephalitis in peninsular Malaysia and Singapore from the autumn of 1998 to the spring of 1999 (Chua *et al.*, 2000). NiV has subsequently been established as the cause of fatal human encephalitis in Bangladesh during 2001, 2003, 2004, 2005, 2007, and 2008 (Banerjee, 2007; Hsu *et al.*, 2004; Promed-mail, 2008; 2004) as well as in India in 2001 and 2007 (Chadha *et al.*, 2006; Promed-mail, 2007c).

Fruit-eating bats of the genus *Pteropus* are a natural reservoir for NiV and HeV (Chua *et al.*, 2002; Halpin *et al.*, 2000; Yob *et al.*, 2001). Humans are infected by exposure to infected fruit bats or material contaminated by infected bats (Hsu *et al.*, 2004), but they are also infected via intermediate hosts such as pigs (Amal *et al.*, 2000; Chew *et al.*, 2000; Paton *et al.*, 1999) or rarely by direct human-to-human contact (Gurley *et al.*, 2007). HeV and NiV cross react with each other antigenically, but not with any of the other paramyxoviruses (Chua *et al.*, 2000). These viruses are the only zoonotic paramyxoviruses that are highly pathogenic in humans, but have a host range that spans five terrestrial mammalian orders, exceeding that of the morbillivirus canine

distemper virus (CDV), which infects species in three orders (Appel & Summers, 1995; Middleton *et al.*, 2002; Mohd Nor *et al.*, 2000; Reynes *et al.*, 2005; Roelke-Parker *et al.*, 1996; Yob *et al.*, 2001). No vaccines or specific antiviral drugs are currently available for NiV. Although patients from the Malaysian outbreak who received ribavirin showed a lower mortality rate (Chong *et al.*, 2001), ribavirin was unable to protect NiV-infected hamsters from fatal disease (Georges-Courbot *et al.*, 2006).

The genomes of NiV and HeV are 18,246 and 18,234 nucleotides (nt) long respectively, making them significantly larger than most other paramyxoviruses, with the exceptions of rodent-borne Beilong and J viruses (Jack *et al.*, 2005; Li *et al.*, 2006). The large genome size is due to a longer open reading frame (ORF) encoding the phosphoprotein (P) and to the large untranslated regions (UTR) that flank each gene (Harcourt *et al.*, 2001; Harcourt *et al.*, 2000; Wang *et al.*, 1998; Wang *et al.*, 2000; Yu *et al.*, 1998a; Yu *et al.*, 1998b). The N, P, and L proteins are required and sufficient for mini-genome replication, similar to other members of the subfamily *Paramyxovirinae* (Halpin *et al.*, 2004). The genus-specific 3' leader and 5' trailer regions serve as promoters for both transcription and replication and are located at the termini of the genome (Lamb & Kolakofsky, 2001), which consists of six genes encoded from 3' to 5', the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), attachment glycoprotein (G), and large (L) polymerase, respectively.

The P genes of the subfamily *Paramyxovirinae* contain several open reading frames (ORFs) in addition to the P ORF. Like most other *Paramyxovirinae*, henipaviruses have a UC-rich region that acts as an editing site to facilitate the non-templated addition of G residues into the transcripts of the P gene mRNA. The additional

G residues allow access to the reading frames of V and W which share amino termini with P, but have unique carboxy termini (Fig. 2A) (Kolakofsky *et al.*, 2005). The C ORF, which is embedded within the P gene and is translated via an alternative translational start site and reading frame, is also present within the P gene of both HeV and NiV. Elegant plasmid-expression studies have been performed to explore viral-host protein interactions and to characterize the functions of the C, V, and W proteins of the henipaviruses (Childs *et al.*, 2007; Ludlow *et al.*, 2008; Park *et al.*, 2003; Rodriguez *et al.*, 2004; Rodriguez *et al.*, 2002; Shaw *et al.*, 2005; Shaw *et al.*, 2004). Both the V and W proteins block interferon (IFN) signaling and induction, while the C protein rescued an IFN-sensitive virus, suggesting that the C protein also inhibits the interferon system. Given the importance of the IFN system in innate immunity, these proteins could be potent virulence factors during infection. In this study, we present the initial characterization of the editing frequencies of the P genes of the henipaviruses, and the first demonstration of the expression of these additional P gene products during NiV infection.

Results

Characterization of NiV and HeV P gene mRNA transcripts

The P genes of HeV and NiV are larger than those of other paramyxoviruses. The length of the open reading frame (ORF) for the P protein is responsible for most of the size increase but the 3' and 5' untranslated regions are also larger (Harcourt *et al.*, 2005; Harcourt *et al.*, 2001; Harcourt *et al.*, 2000; Wang *et al.*, 2000). Based on genome sequence analysis, the predicted size of the mRNAs produced from the P genes of HeV and NiV are 2,698 nt and 2,704 nt, respectively, though these transcripts have yet to be characterized from infected cells. Northern blot analysis verified that the longer untranslated regions are incorporated into P mRNAs from the Malaysian strain of NiV (NiV-M) and HeV infected cells, as the mRNA transcripts were specifically recognized by the HeV or NiV-M probes, and migrated with the size predicted by the sequence analysis (Figure 1).

Determination of P gene mRNA editing frequencies for NiV and HeV

To determine the mRNA editing frequency of the P genes of the henipaviruses, RT-PCR was performed to amplify the editing sites of HeV, NiV-M, and the Bangladesh strain of NiV (NiV-B). The PCR products were separately cloned into the plasmid cloning vector, pCR2.1 TA, approximately 100 plasmid clones were isolated for each PCR product, and the nucleotides containing the henipavirus editing sites in each plasmid were sequenced. When similar techniques were used to analyze the P gene transcripts of other paramyxoviruses such as MeV, two-thirds of the mRNAs transcribed from the P gene were not edited, and approximately 90% of the edited mRNAs had a single non-

templated G residue added (Bankamp *et al.*, 2008; Vanchiere *et al.*, 1995)(Figure 2C). In contrast, only one-third of the henipavirus transcripts were not edited. The majority of transcripts were edited, 67% in NiV-M, 66% in NiV-B, and 71% in HeV (Figure 2B). The edited henipavirus transcripts were distributed from those with 1 inserted G (13%) through though those with 7 inserted G residues (5%), and a few had larger inserts of up to 14 G residues (1%). Though it is not known how the addition of a GGG triplet encoding a glycine residue or multiple glycine residues in the any of these proteins might alter its function, it was presumed that transcripts with 3, 6, or 9 G residues added would encode a functional P protein. Similarly, transcripts with 4, 7, or 10 G residues would encode a functional V protein, and those with 5, 8, 11 or 14 Gs would encode a functional W protein. If all of the proteins with inserted glycines are functional, nearly 50% of all transcripts from the P gene of the henipaviruses would encode the P protein, 25% would encode the V protein, and 25% would encode the W protein (Figure 2C).

Mouse antipeptide sera against NiV P, V, W, and C specifically detect their respective proteins

Radioimmunoprecipitations (RIPs) of transfected 293 cells individually expressing the P, C, V, or W protein confirmed that the monospecific antiserum made against each of these proteins recognized the correct protein (Figure 3). The C protein, predicted to be 19.7 kDa, migrated at an apparent weight of 20 kDa. The V and W proteins migrated at an apparent molecular weight of 55 kDa, slightly larger than the predicted 50.6 kDa. This was also observed for the P protein, which is predicted to be 78.3 kDa, but migrated at an apparent molecular weight of 80-85 kDa (Shiell *et al.*,

2003). RIPs of lysates from Vero cells transfected with the P, V, or W-expressing plasmids precipitated with PVW antisera (Figure 3, right 3 lanes) confirmed that the migration of each protein was similar to that shown in 293 cells (Figure 3, left lanes). Taken together, these results show that the antipeptide antiserum developed against the P, C, V, and W proteins correctly detected their respective proteins.

Detection of NiV P, C, V, and W in virus-infected cells and virions

To confirm the presence of NiV P, C, V, and W in NiV-infected cells, Western blots were performed on NiV-infected Vero cells (Figure 4A, odd numbered lanes). Due to the high level of homology between the NiV peptide sequences utilized to generate the antipeptide antisera and their corresponding sequences in HeV (P- 80%, V- 85%, W- 80%, C- 62%), HeV-infected cell lysates were also tested (Figure. 4A, even numbered lanes). The respective antisera specific for NiV V and W each cross-reacted with the corresponding V and W proteins of HeV. The bands corresponding to HeV V and W were less intense than the bands for NiV V and W probably because the growth rate of HeV is slower than that of NiV, resulting in a lower level of HeV protein expression in cell lysates at 36 hr post-infection. The antiserum to NiV C detected a protein with an apparent molecular weight of approximately 16 kDa, slightly smaller than the expected size of C. The antiserum to NiV C did not react with any protein in the HeV-infected lysate, and the antiserum to NiV P did not react with a protein migrating at the expected size of HeV P, although the band detected in the lysates from HeV could be a degradation product of HeV P (Figure. 4A). None of the antisera recognized proteins in the mock-

infected cell lysates. These results indicate that the P, C, V, and W proteins of NiV are present in infected cells.

Sucrose gradient-purified NiV virions were examined by Western blot to determine which of the P, C, V, and W proteins were present. All of these proteins were detected and each protein migrated with the same apparent molecular mass as the proteins detected in lysates of infected cells (Figure. 4B). Compared to the Western blots of crude infected cell lysates (Figure. 4A), the P protein band detected in the purified NiV was markedly more abundant than the C, V, and W bands, and the putative degradation products were not present (Figure. 4B). The antiserum to NiV C detected a minor amount of an additional protein which had an electrophoretic mobility similar to that of NiV P. These results indicate the presence of P, C, V, and W proteins in the purified virions.

NiV P, V, W and C are distinctly distributed in the cell

To determine the cellular location of NiV P, V, W, and C in NiV-infected cells, immunofluorescence assays were performed using the specific mouse antipeptide antisera. The P protein was detected throughout the cell and did not co-localize with the nucleus, but appeared to be concentrated at the plasma membrane (Figure 5). A similar distribution was found for the N protein. The C protein was detected throughout the cytoplasm of the cell in a punctate pattern and was visibly excluded from the nucleus. The V protein was also found exclusively in the cytoplasm, but the distribution was more uniform than that of C, resulting in a pronounced boundary between the nucleus and the cytoplasm. In contrast, the W protein was detected exclusively in the nucleus, and was

distributed relatively evenly throughout it, with the exception of the nucleoli. These results demonstrate that the P, C, V, and W proteins are distinctly distributed in an infected cell.

KPNA3 coprecipitates with NiV W protein in infected cell lysates

In order to determine whether NiV W interacts with KPNA3 during viral infection, co-immunoprecipitations and western blots were performed using mouse antiserum against NiV W and goat antiserum against KPNA3. When the antiserum against NiV W was used to probe lysates immunoprecipitated with either NiV antiserum or KPNA3 antiserum, it was shown that W protein was detected (Figure 6), while a lysate immunoprecipitated with an antibody against STAT-1 did not yield any detectable amount of W protein by western blot. These results indicate that KPNA3 interacts with NiV W protein in the context of a viral infection.

Discussion

Viral polymerase-dependent mRNA editing has long been documented as a distinctive feature of viruses in the family *Paramyxoviridae*, subfamily *Paramyxovirinae*. In this study, mRNA editing by henipaviruses occurred in approximately two-thirds of all P gene transcripts. Therefore, the henipaviruses produced edited transcripts at twice the frequency of the other viruses (Bankamp *et al.*, 2008; Hausmann *et al.*, 1999; Kato *et al.*, 1997; Mebatsion *et al.*, 2003; Vanchiere *et al.*, 1995) with the exception of HPIV 3, which edits approximately half of its P gene transcripts, and bovine parainfluenza virus 3 (BPIV3), which edits mRNA to yield proteins from all 3 ORFs at a similar frequency to what was found in this report for NiV (Galinski *et al.*, 1992; Pelet *et al.*, 1991).

Mutational studies of the editing sites of the P genes of several paramyxoviruses have identified important nucleotide positions that play a role in viral editing frequency. For Sendai virus, it was shown that a 6-8 nucleotide region upstream of the A_nG_n sequence can significantly modify mRNA editing frequency (Delenda *et al.*, 1998; Hausmann *et al.*, 1999). Making a C-U mutation at the nucleotide proximal to the A_nG_n in Newcastle disease virus altered editing frequencies to yield more transcripts coding for V and W than those coding for P protein (Mebatsion *et al.*, 2003). The editing site sequences of NiV-M and HeV are identical to that of measles virus (MeV) (GGGUAAUUUUUCCCGUGUC), but the Bangladesh Nipah isolate (NiV-B) differs by one nucleotide (underlined), (GGGAUAAUUUUUCCCGUGUC). Although the A residue appears to be an insertion, the hexameric phase of the editing site remains the same as that of NiV-M (Harcourt *et al.*, 2005). Based on the nucleotide sequences within the editing site, it would be predicted that the editing frequencies of MeV and the

henipaviruses would be similar. However, the differences between the editing frequencies of the henipaviruses and MeV suggest the possibility of other determinants including other cis-acting sequences or the L protein. Compared to other non-segmented single-stranded RNA viruses, the putative catalytic site (GDNE) of the NiV polymerase is more tolerant of mutations in the last residue GDNX, which suggests that the catalytic mechanism of the L protein of NiV might be slightly different (Magoffin *et al.*, 2007).

This study provides the first documentation of the presence and the cellular distributions of P, V, W, and C proteins in NiV-infected cells. The cellular distributions of V and W proteins are consistent with previous studies in which the V and W ORFs were individually expressed via plasmids in mammalian cells (Rodriguez & Horvath, 2004; Rodriguez *et al.*, 2002; Shaw *et al.*, 2005; Shaw *et al.*, 2004). The speckled distribution of the P protein within the cytoplasm of NiV-infected cells and its accumulation near the plasma membrane is in contrast to the homogenous cytoplasmic distribution shown in previous studies in which the P ORF was expressed individually in mammalian cells (Shaw *et al.*, 2004). The P proteins of paramyxoviruses interact with both the L protein and the N protein-encapsulated genome, so it is likely that the specific interactions of P with these proteins could affect its observed localization within NiV-infected cells (Bankamp *et al.*, 1996; Curran *et al.*, 1995; Curran *et al.*, 1994; Horikami *et al.*, 1992; Horikami *et al.*, 1994). As expected, the distribution of the NiV N protein was similar to that of NiV P. The interaction domains of the NiV N and P proteins have been mapped (Chan *et al.*, 2004). The cellular distribution of the NiV P and V proteins in this study are similar to those observed for the P and V proteins of MeV and rinderpest virus (Sweetman *et al.*, 2001; Wardrop & Briedis, 1991).

In this study, we detected C, V, and W along with P in preparations of purified NiV virions. Incorporation of V or C proteins into virions is not common among viruses in the subfamily *Paramyxovirinae*. While the presence of V protein has been demonstrated in the virions of SV5, mumps virus, and HeV, they have not been detected in MeV or SeV (Curran *et al.*, 1991; Kato *et al.*, 1997; Paterson *et al.*, 1995; Shiell *et al.*, 2003; Takeuchi *et al.*, 1990). However, the C protein has been detected in MeV and SeV virions (Devaux & Cattaneo, 2004; Yamada *et al.*, 1990). It is possible that cytoplasmic or membranous contamination in the NiV purified virion preparation may have resulted in the detection of V, W, and C. However, the absence of V and W protein degradation products that are detected in the cell lysates argues against the V and W proteins in the virions being contaminants from cell lysates. Granted that the anti-C serum detected a protein with a similar electrophoretic mobility to the P protein, this may indicate that the C associates with P in virions.

The C, V, and W proteins of NiV inhibit both transcription and replication in a NiV minigenome assay (Sleeman *et al.*, 2008). The distinct cellular distribution of each protein shown in the present study implies that different mechanisms underlie the inhibition caused by each of these proteins. For example, in cells infected with MeV, a specific interaction between C and L proteins is required in order to inhibit viral RNA transcription and replication, while the inhibition mediated by the V protein correlates specifically with its ability to bind RNA (Bankamp *et al.*, 2005; Grogan & Moyer, 2001; Parks *et al.*, 2006; Smallwood & Moyer, 2004; Witko *et al.*, 2006). Since the W protein localizes to the nucleus, the mechanism behind its inhibition of replication is likely different from that of C and V, as it is unlikely that it interacts with the cytoplasmic viral

polymerase. A number of plasmid expression studies have demonstrated various interactions between NiV P, V and W proteins with certain host cell proteins including STAT-1 (P, V and W), mda-5 (V), and karyopherin α (KPNA) 3 and 4 (W). These interactions were all linked to the ability of the P, V, and W proteins to block IFN signaling and/or IFN induction (Childs *et al.*, 2007; Rodriguez *et al.*, 2002; Rodriguez *et al.*, 2003; Shaw *et al.*, 2005; Shaw *et al.*, 2004). STAT-1 is a molecule crucial to both the type I and type II IFN signaling pathways (Bronze & Greenfield, 2003). Mda-5 is an RNA helicase known to stimulate production of IFN-β upon detecting double-stranded RNA (Kang *et al.*, 2002). KPNA3 is part of the nuclear import machinery that recognizes cargo protein by binding to its nuclear localization sequence (Chook & Blobel, 2001). Since the W and the IFN regulatory factor 3 (IRF-3) proteins share an affinity for KPNA3 (Kumar *et al.*, 2000), W competes with IRF-3 for binding, thus preventing activated IRF-3 translocation into the nucleus. Another host protein PLK-1 has recently been shown to interact with the V and W proteins independently of the STAT-1 protein interaction (Ludlow *et al.*, 2008). While it was demonstrated that PLK-1 binding did not affect NiV minigenome replication, the implications of this interaction have yet to be determined in NiV infection.

This is the first study in which the expression and cellular distribution of the NiV C protein has been characterized in NiV infected cells. Plasmid-expressed NiV C rescued an interferon-sensitive NDV, but the mechanism behind this effect remains undefined (Park *et al.*, 2003). Studies to identify host proteins that interact with C merit further investigation. The C proteins of paramyxoviruses inhibit antiviral responses, and serve as virulence factors (Devaux & Cattaneo, 2004; Devaux *et al.*, 2008; Escoffier *et*

al., 1999; Garcin *et al.*, 2001; Gotoh *et al.*, 2001; Nakatsu *et al.*, 2006; Nakatsu *et al.*, 2008; Takeuchi *et al.*, 2005). This study is also the first demonstration of NiV W expression and localization in infected cells, and we have thus far confirmed the interaction between W and KPNA3 in NiV-infected cells by co-immunoprecipitation and western blot (Figure 6). With the availability of a full-length NiV reverse genetic system, further investigation of the roles of C, V, and W in viral replication and pathogenesis is now possible (Yoneda *et al.*, 2006). A recent report indicating that NiV can cause immunosuppression in pigs, suggests that understanding the impact of C, V, and W proteins during NiV infection *in vivo* will be key to understanding the molecular mechanisms of pathogenesis (Berhane *et al.*, 2008).

Materials and Methods

Cells and Viruses

Vero E6, Vero, and 293 cells were maintained in Dulbeco's Modified Essential Medium (DMEM) containing 10% fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin (GIBCO BRL). The Nipah virus Malaysia (NiV-M) strain used in this study was isolated directly from human brain tissue (Chua *et al.*, 2000). The Nipah virus Bangladesh strain (NiV-B) was isolated from an oropharyngeal sample from a patient from Rajbari (Harcourt *et al.*, 2005), and the Hendra virus (HeV) used was the isolate obtained from the first fatal human case of HeV (Murray *et al.*, 1995b). The viruses were passaged in Vero E6 cells and harvested when the cytopathic effect (CPE) was maximal. All work with live virus was performed under biosafety level 4 (BSL-4) conditions in either the maximum containment laboratory at CDC, Atlanta, GA, USA or in the BSL-4 laboratory at the Australian Animal Health Laboratory at CSIRO, Geelong,Victoria, Australia.

RNA extraction, RT-PCR, cloning and sequencing of P gene mRNA transcripts

For cloning the editing sites, RNA was extracted from infected cell lysates (Qiagen), and poly-A-plus RNA was purified with a Promega Oligo dT Kit. RT-PCR was performed as previously described (Rota *et al.*, 1994) except that the PCR cycling conditions were 95 °C for 3 min., followed by 35 cycles of 95 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min. The reaction was then incubated at 72 °C for an additional 5 min. PCR products were visualized using agarose gel electrophoresis and were purified

using the Wizard PCR Preps DNA Purification System (Promega). The primers used for amplification of an entire P gene including the editing site were as follows: HeV P1 (forward), 5'- ATGGACAAGTTGGATCTAGTC-3' and HeV P16 (reverse), 5'-TTAGATGTTCCCATCAATAATATC-3' [P gene of HeV]; NiV P1 (forward), 5'-ATGGATAAATTGGAACTAGTC-3' and NiV P18 (reverse), 5'-TCAAATATTACCGTCAATGATG-3' [P gene of NiV-M]; and NiVB PFC (forward) 5'-ATGGATAAATTGGAACTAGTT-3' and NiVB PRC (reverse) 5'-TCAAATGTTACCGTCAATGATG-3 [P gene of NiV-B]. Purified PCR products were cloned into PCR2.1 using the TA Cloning Kit (Invitrogen) following the manufacturer's instructions. White colonies were picked, grown in 5 ml of LB broth, and the plasmid DNA was isolated using a MiniPrep Kit (Qiagen). Approximately 100 colonies containing the P gene amplicon of HeV, NiV-M, and NiV-B were sequenced using a cycle sequencing reaction with fluorescent dye terminators (Perkin-Elmer, Applied Biosystems Division), and the reaction products were analyzed by using ABI 373 and 3100 automatic sequencers (Perkin-Elmer, Applied Biosystems Division). The primers used for sequencing the editing sites were as follows: HeV P8 (forward) 5'-TGATCCAATAATACAAGAATT-3', HeV P13 (reverse) 5'-CATTGAGATGGTCTGCATGATA-3', NiV P8 (forward) 5'-TGAGTGCTCTGGATCGGAAGA-3', and NiV P13 (reverse) 5'-AATGATCTGCGTGATAATTCA-3'.

NiV and HeV P gene probes for northern blot

Specific digoxigenin (DIG)-labeled NiV Malaysia (NiV-M) and HeV P genes probes were synthesized by PCR using primer-pairs to amplify the full P gene with a DIG-labeling kit (Roche), using corresponding clones of the P genes in the TA cloning vector PCR2.1 (Invitrogen) as template. Five µg of total RNA from mock, NiV, or HeV infected Vero cells in 5 µl volume were heated at 65°C for 10 min in the presence of 1 µl RNA E buffer, 3.5 µl 37% formaldehyde, 10 µl formamide and 3 µl loading buffer. The samples were put in an ice bath for 3 min, centrifuged briefly in a microcentrifuge, and electrophoresed in 2% agarose formaldehyde gel at 30 volts for 17 hr in MOPS EDTA acetate buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5). Following electrophoresis, the gel was transferred to a nylon membrane using a vacuum transfer apparatus for 3 hrs before being cross-linked using the AUTO setting on a UV Stratalinker 1800 (Stratagene). Steps of prehybridization, hybridization to the DIG-labeled P gene of NiV-M or HeV probes (250 ng ml^{-1}), washings and subsequent detection with chemiluminescent substrate (CDP-star) were performed according to the manufacturer instructions (Roche). The membrane was then exposed to X-ray film for various exposure times.

Development of antipeptide sera against NiV P, C, V, and W

Peptides were designed from each predicted protein within the P gene: P, C, V, and W, using the sequences from strain NiV-M (GenBank accession number AF212302) and synthesized by the CDC Core Laboratory Facility. The peptides used were as follows: P (amino acids 411-450), CKYPSAGTENVPGSKSGATRHVRGSPPYQEGKSVNAENVQL; V (amino acids

411-450), CEISICWDGKRAWVEEWCNPACSRITPLPRRQECQCGECPT; W (amino acids 409-448), CAQTRNIHLLGRKTCLGRRVVQPGMFEDHPPTKKARVSMRR; and C (amino acids 19-58), CRHTDDQVFNNPASKIKQKPGKIFCSAPVENLNKLRGECLR. A cysteine was added to the amino terminus of each peptide to provide a sulfhydryl group to facilitate conjugation. A peptide consisting of the shared amino acids on the amino terminal side of the editing site was used to create an antibody capable of detecting P, V, and W (PVW peptide). The PVW peptide was made commercially (Invitrogen) and consisted of amino acids 381-400, NGAVQTADRQRPGTPMPKSR. The P, C, and W peptides were conjugated to the carrier protein, keyhole limpet hemocyanin (KLH) using the Imject Maleimide Activated Immunogen Conjugation Kit with mcKLH and BSA (Pierce) following the manufacturer's instructions. Since the V peptide was insoluble in the kit's conjugation buffer, even when supplemented with 30% DMSO, an alternative protocol adapted from Current Protocols in Immunology was used (Grant, 2003).

The V peptide was coupled to KLH using the following protocol. Five mg of KLH (Pierce) was dissolved in 0.5 ml 0.01 M phosphate buffer, pH 7.0 and dialyzed against 4 liters of 0.01 M phosphate buffer, pH 7.0 overnight at 4°C with stirring using a 0.5 ml to 3.0 ml Slide-A-Lyzer Dialysis Cassette with a 10,000 MWCO (Pierce). Dialyzed protein was transferred to a small glass beaker and 70 ml of 15 mg/ml *m*-maleimidobenzoly-*N*-hydrosuccinimide ester (Pierce) in dimethylformamide (MBS/DMF) prepared within 1 hour of use was added and stirred gently for 30 min at RT. A PD-10 column (GE Life Sciences, Pittsburgh, PA) was equilibrated with 50 ml 0.05 M phosphate buffer, pH 6.0, before loading the KLH/MBS/DMF reaction mixture.

The column was eluted with 0.05 M phosphate buffer, pH 6.0, and 20 0.5 ml fractions were collected and the A_{280} read on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). The first peak represented the MB/KLH conjugate (fractions 4 and 5), and was pooled into a 15-ml test tube. Five milligrams of V synthetic peptide were dissolved in 1.0 ml of 6 M guanidine-HCL/0.01 M phosphate buffer, pH 7.0, and added to the MB/KLH conjugate. The pH of the mixture was adjusted to 7.3 using 0.1 M HCL or 0.1 M NaOH and then stirred for 3 hr at RT with a magnetic stir bar. The mixture was then dialyzed against 4 liters of water overnight at 4°C using a 0.5 ml to 3.0 ml Slide-A-Lyzer Dialysis Cassette with a 10,000 MWCO. The water was replaced with fresh water and dialyzed for ≥4 hr at 4°C. Protein concentrations were determined using the BCA Protein Assay (Pierce).

Ten BALB\c mice were inoculated once intraperitoneally (IP) with 50 µg of conjugated protein with TiterMax Gold adjuvant (Sigma-Aldrich) and three times with 50 µg of conjugated protein with Freund's Incomplete adjuvant (Sigma-Aldrich) in two-week intervals. Two weeks after the final inoculation, the mice were anesthetized and exsanguinated and the serum for each peptide antibody was pooled. The PVW peptide was conjugated to the carrier protein, keyhole limpet hemocyanin (KLH), and used to immunize two rabbits four times over a ten week time period by Invitrogen. Serum was taken at ten weeks post inoculation.

Screening antipeptide sera by radioimmunoprecipitation

Approximately 5 x 10^5 293 cells/well in a 6-well plate were transfected with 4 µg of a eukaryotic expression plasmid pCAGGS (Niwa *et al.*, 1991) containing the ORF of

either NiV P, C, V, W, or empty vector using LT-1 transfection reagent (Mirusbio). For the P, V, and W expression plasmids, the start codons in the alternative C ORF were changed from 'ATG' to 'ACG' to prevent co-expression of C protein. At 24 hr post-transfection, cells were starved for 1 hr in DMEM lacking in methionine (GIBCO BRL), and then each well was supplemented with ~10 μCi of S^{35}-Methionine (Amersham Biosciences) for 2 hr, washed with PBS, and lysed in 500 μL of RIPA buffer (150 mM NaCl, 50mM Tris-HCl, pH 7.5, 1% Sodium deoxycholate, 1% Triton X-100, 0.2% SDS). Cell lysates were diluted 1:5 and incubated with 1 μl of normal mouse serum for 1 hr, and then incubated with Protein G sepharose beads (Amersham Biosciences) for 20 min to precipitate mouse IgG. The beads were centrifuged for 1 min at 5000 rpm, and the supernatants were incubated with 3 ul of P, C, V, or W antiserum overnight at 4°C. These samples were subsequently incubated with 293 cell lysate-tumbled Protein G sepharose beads for 20 min to precipitate the IgG. The supernatants were discarded, the beads were washed 3X with RIPA buffer, and resuspended in 30 μl of SDS PAGE running buffer containing 1X Laemmli sample buffer and 2 μl of β-mercaptoethanol. These samples were then boiled for 5 min and electrophoresed on a denaturing 12% SDS polyacrylamide gel (Bio-Rad). The radioactive signal was amplified by treating the gel with Amplify fluorographic reagent (Amersham Biosciences). The gel was dried onto blotting paper (Bio-Rad), incubated with Xomat film (Kodak) overnight, and exposed the following day. As a control, Vero cells transfected with pCAGGS-P, pCAGGS-V, or pCAGGS-W were used as antigen in conjunction with the PVW antipeptide antiserum.

Western Blot and immunoprecipitations of infected cell lysates and purified virus antigen

Approximately 5 x 10^5 Vero cells per well of a 6-well plate were infected with NiV or HeV at an MOI of 1, and harvested in 500 µl of RIPA buffer at 36 hr, denatured with 2% SDS and 50 µM DTT, and boiled for 10 min. Six µl of sample was run on a denaturing 4-12% SDS-PAGE gel (Novex). Proteins were transferred from the gel onto polyvinilidene fluoride (PVDF) membranes, which were blocked overnight in TBS-T (200mM NaCl, 50mM Tris-HCl pH 7.4, 0.5% Tween) with 5% skim milk. PVDF membranes were incubated with P, C, V, or W antiserum diluted in TBS-T with 5% milk (working dilution from 1:400 for P, C, V to 1:4000 for W) for 1 hr at room temperature, and washed 3 times in TBS-T. The membranes were then incubated with a goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) (1:10,000 dilution) (Ebioscience) for 1 hr at RT, washed 3X in TBS-T, incubated with ECL reagent (Promega) for 1 min, and exposed to film for development. For sample loading controls, the blots were stripped with Reblot Plus Strong Antibody Stripping Solution (Chemicon), blocked with TBS-T with 5% milk for 1 hr, washed 3X in TBS-T, incubated with rabbit anti-β-Actin primary antibody (1:2,000) for 1 hr, and washed 3X in TBS-T. The membranes were then incubated with anti-rabbit IgG conjugated with HRP for 1 hr (1:10,000), washed 3X in TBS-T, incubated with ECL reagent, and exposed to film as stated above. For Western blots using sucrose gradient-purified virus antigen, 2 µl of sample was used for each blot against P, C, V, and W. Immunoprecipitation of NiV W from infected Vero cell lysates was performed as stated in the above manner with lysates expressing W protein from a eukaryotic plasmid. A goat antibody against Karyopherin alpha 3 (KPNA3) was obtained from Imgenex, and 3 µL was used per mL of infected Vero cell lysates to immunoprecipitate KPNA3. Ensuing Western blot performed to

detect W protein was executed as mentioned above.

Preparation of purified NiV antigen

Confluent Vero cells were inoculated with NiV in 850 cm^2 roller bottles for 30 min at 33°C. After adsorption for 30 min at 33°C, growth media was added to each roller bottle and rolled for 48 hrs at 33°C. The cells were observed daily for 2-3 days, and the supernatant were harvested and pooled when optimal levels of CPE were reached. The cell debris was removed by centrifugation at 10,000 g for 10 min, with the remaining supernatant being subsequently centrifuged at 50,000 rpm for 20 min at 8°C. The supernatant from the subsequent centrifugation was removed, and the pellet was resuspended overnight in 70 µl of TNE buffer (0.01 M Tris–HCl pH 7.5; 0.1 M NaCl; 1 mM EDTA) at 4°C. The viral pellet was loaded onto a 20% - 50% sucrose continuous gradient buffered in 20mM Tris-HCl pH 7.6, and centrifuged at 26,000 rpm (SW41 rotor, Beckman J6 ultracentrifuge) for 30 min. The viral band was harvested by needle and syringe, diluted in TNE, and then centrifuged for 40,000 rpm (SW41 rotor, Beckman J6 ultracentrifuge) for 1 hr to pellet the virus. The supernatant was discarded and the viral pellet was resuspended in 1-2ml of 2% SDS, and boiled for 2 min.

Immunofluorescence Assay

Vero cells in chamber slides (40,000 cells/chamber) were infected with NiV at an MOI of 0.01 for 24-36 hr at 37°C, fixed in 100% cold methanol, blocked with PBS-Tween (PBS + .05% tween) in 2% milk for 30 min, stained for another 30 min with primary antiserum against NiV P, C, V, W, or N (Aljofan *et al.*, 2008) and washed 3X

with PBS-T. Cells were then concurrently stained with DAPI and a goat anti-mouse conjugated with Alexaflour 488 (Sigma) for 30 min, washed 3X with PBS-T, and then examined under the microscope to detect fluorescent cells, indicating the presence and cellular localization of the respective viral antigens.

References

Aljofan, M., Porotto, M., Moscona, A. & Mungall, B. A. (2008). Development and validation of a chemiluminescent immunodetection assay amenable to high throughput screening of antiviral drugs for Nipah and Hendra virus. *J Virol Methods* **149**, 12-19.

Amal, N. M., Lye, M. S., Ksiazek, T. G., Kitsutani, P. D., Hanjeet, K. S., Kamaluddin, M. A., Ong, F., Devi, S., Stockton, P. C., Ghazali, O., Zainab, R. & Taha, M. A. (2000). Risk factors for Nipah virus transmission, Port Dickson, Negeri Sembilan, Malaysia: results from a hospital-based case-control study. *Southeast Asian J Trop Med Public Health* **31**, 301-306.

Appel, M. J. & Summers, B. A. (1995). Pathogenicity of morbilliviruses for terrestrial carnivores. *Vet Microbiol* **44**, 187-191.

Banerjee, R. (2007). Drug for bat virus combat. In *The Telegraph*. Calcutta, India.

Bankamp, B., Horikami, S. M., Thompson, P. D., Huber, M., Billeter, M. & Moyer, S. A. (1996). Domains of the measles virus N protein required for binding to P protein and self-assembly. *Virology* **216**, 272-277.

Bankamp, B., Lopareva, E. N., Kremer, J. R., Tian, Y., Clemens, M. S., Patel, R., Fowlkes, A. L., Kessler, J. R., Muller, C. P., Bellini, W. J. & Rota, P. A. (2008). Genetic variability and mRNA editing frequencies of the phosphoprotein genes of wild-type measles viruses. *Virus Res* **135**, 298-306.

Bankamp, B., Wilson, J., Bellini, W. J. & Rota, P. A. (2005). Identification of naturally occurring amino acid variations that affect the ability of the measles virus C protein to regulate genome replication and transcription. *Virology* **336**, 120-129.

Berhane, Y., Weingartl, H. M., Lopez, J., Neufeld, J., Czub, S., Embury-Hyatt, C., Goolia, M., Copps, J. & Czub, M. (2008). Bacterial infections in pigs experimentally infected with nipah virus. *Transbound Emerg Dis* **55**, 165-174.

Bronze, M. S. & Greenfield, R. A. (2003). Preventive and therapeutic approaches to viral agents of bioterrorism. *Drug Discov Today* **8**, 740-745.

Chadha, M. S., Comer, J. A., Lowe, L., Rota, P. A., Rollin, P. E., Bellini, W. J., Ksiazek, T. G. & Mishra, A. (2006). Nipah virus-associated encephalitis outbreak, Siliguri, India. *Emerg Infect Dis* **12**, 235-240.

Chan, Y. P., Koh, C. L., Lam, S. K. & Wang, L. F. (2004). Mapping of domains responsible for nucleocapsid protein-phosphoprotein interaction of Henipaviruses. *J Gen Virol* **85**, 1675-1684.

Chew, M. H., Arguin, P. M., Shay, D. K., Goh, K. T., Rollin, P. E., Shieh, W. J., Zaki, S. R., Rota, P. A., Ling, A. E., Ksiazek, T. G., Chew, S. K. & Anderson, L. J. (2000). Risk factors for Nipah virus infection among abattoir workers in Singapore. *J Infect Dis* **181**, 1760-1763.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R. & Goodbourn, S. (2007). mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* **359**, 190-200.

Chong, H. T., Kamarulzaman, A., Tan, C. T., Goh, K. J., Thayaparan, T., Kunjapan, S. R., Chew, N. K., Chua, K. B. & Lam, S. K. (2001). Treatment of acute Nipah encephalitis with ribavirin. *Ann Neurol* **49**, 810-813.

Chook, Y. M. & Blobel, G. (2001). Karyopherins and nuclear import. *Curr Opin Struct Biol* **11**, 703-715.

Chua, K. B., Bellini, W. J., Rota, P. A., Harcourt, B. H., Tamin, A., Lam, S. K., Ksiazek, T. G., Rollin, P. E., Zaki, S. R., Shieh, W., Goldsmith, C. S., Gubler, D. J., Roehrig, J. T., Eaton, B., Gould, A. R., Olson, J., Field, H., Daniels, P., Ling, A. E., Peters, C. J., Anderson, L. J. & Mahy, B. W. (2000). Nipah virus: a recently emergent deadly paramyxovirus. *Science* **288**, 1432-1435.

Chua, K. B., Koh, C. L., Hooi, P. S., Wee, K. F., Khong, J. H., Chua, B. H., Chan, Y. P., Lim, M. E. & Lam, S. K. (2002). Isolation of Nipah virus from Malaysian Island flying-foxes. *Microbes Infect* **4**, 145-151.

Curran, J., de Melo, M., Moyer, S. & Kolakofsky, D. (1991). Characterization of the Sendai virus V protein with an anti-peptide antiserum. *Virology* **184**, 108-116.

Curran, J., Marq, J. B. & Kolakofsky, D. (1995). An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. *J Virol* **69**, 849-855.

Curran, J., Pelet, T. & Kolakofsky, D. (1994). An acidic activation-like domain of the Sendai virus P protein is required for RNA synthesis and encapsidation. *Virology* **202**, 875-884.

Delenda, C., Taylor, G., Hausmann, S., Garcin, D. & Kolakofsky, D. (1998). Sendai viruses with altered P, V, and W protein expression. *Virology* **242**, 327-337.

Devaux, P. & Cattaneo, R. (2004). Measles virus phosphoprotein gene products: conformational flexibility of the P/V protein amino-terminal domain and C protein infectivity factor function. *J Virol* **78**, 11632-11640.

Devaux, P., Hodge, G., McChesney, M. B. & Cattaneo, R. (2008). Attenuation of V- or C-defective measles viruses: infection control by the inflammatory and interferon responses of rhesus monkeys. *J Virol* **82**, 5359-5367.

Escoffier, C., Manie, S., Vincent, S., Muller, C. P., Billeter, M. & Gerlier, D. (1999). Nonstructural C protein is required for efficient measles virus replication in human peripheral blood cells. *J Virol* **73**, 1695-1698.

Galinski, M. S., Troy, R. M. & Banerjee, A. K. (1992). RNA editing in the phosphoprotein gene of the human parainfluenza virus type 3. *Virology* **186**, 543-550.

Garcin, D., Curran, J., Itoh, M. & Kolakofsky, D. (2001). Longer and shorter forms of Sendai virus C proteins play different roles in modulating the cellular antiviral response. *J Virol* **75**, 6800-6807.

Georges-Courbot, M. C., Contamin, H., Faure, C., Loth, P., Baize, S., Leyssen, P., Neyts, J. & Deubel, V. (2006). Poly(I)-poly(C12U) but not ribavirin prevents death in a hamster model of Nipah virus infection. *Antimicrob Agents Chemother* **50**, 1768-1772.

Gotoh, B., Komatsu, T., Takeuchi, K. & Yokoo, J. (2001). Paramyxovirus accessory proteins as interferon antagonists. *Microbiol Immunol* **45**, 787-800.

Grant, G. (2003). Unit 9.2 Synthetic Peptides for Production of Antibodies that Recognize Intact Proteins In *Current Protocols in Immunology*

pp. Unit 9.2.9, Supplement 55. Edited by R. Coico. Hoboken: John Wiley and Sons Inc

Grogan, C. C. & Moyer, S. A. (2001). Sendai virus wild-type and mutant C proteins show a direct correlation between L polymerase binding and inhibition of viral RNA synthesis. *Virology* **288**, 96-108.

Gurley, E. S., Montgomery, J. M., Hossain, M. J., Bell, M., Azad, A. K., Islam, M. R., Molla, M. A., Carroll, D. S., Ksiazek, T. G., Rota, P. A., Lowe, L., Comer, J. A., Rollin, P., Czub, M., Grolla, A., Feldmann, H., Luby, S. P., Woodward, J. L. & Breiman, R. F. (2007). Person-to-person transmission of Nipah virus in a Bangladeshi community. *Emerg Infect Dis* **13**, 1031-1037.

Halpin, K., Bankamp, B., Harcourt, B. H., Bellini, W. J. & Rota, P. A. (2004). Nipah virus conforms to the rule of six in a minigenome replication assay. *J Gen Virol* **85**, 701-707.

Halpin, K., Young, P. L., Field, H. E. & Mackenzie, J. S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J Gen Virol* **81**, 1927-1932.

Hanna, J. N., McBride, W. J., Brookes, D. L., Shield, J., Taylor, C. T., Smith, I. L., Craig, S. B. & Smith, G. A. (2006). Hendra virus infection in a veterinarian. *Med J Aust* **185**, 562-564.

Harcourt, B. H., Lowe, L., Tamin, A., Liu, X., Bankamp, B., Bowden, N., Rollin, P. E., Comer, J. A., Ksiazek, T. G., Hossain, M. J., Gurley, E. S., Breiman, R. F., Bellini, W. J. & Rota, P. A. (2005). Genetic characterization of Nipah virus, Bangladesh, 2004. *Emerg Infect Dis* **11**, 1594-1597.

Harcourt, B. H., Tamin, A., Halpin, K., Ksiazek, T. G., Rollin, P. E., Bellini, W. J. & Rota, P. A. (2001). Molecular characterization of the polymerase gene and genomic termini of Nipah virus. *Virology* **287**, 192-201.

Harcourt, B. H., Tamin, A., Ksiazek, T. G., Rollin, P. E., Anderson, L. J., Bellini, W. J. & Rota, P. A. (2000). Molecular characterization of Nipah virus, a newly emergent paramyxovirus. *Virology* **271**, 334-349.

Hausmann, S., Garcin, D., Morel, A. S. & Kolakofsky, D. (1999). Two nucleotides immediately upstream of the essential A6G3 slippery sequence modulate the pattern of G insertions during Sendai virus mRNA editing. *J Virol* **73**, 343-351.

Hooper, P. T. & Williamson, M. M. (2000). Hendra and Nipah virus infections. *Vet Clin North Am Equine Pract* **16**, 597-603,.

Horikami, S. M., Curran, J., Kolakofsky, D. & Moyer, S. A. (1992). Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *J Virol* **66**, 4901-4908.

Horikami, S. M., Smallwood, S., Bankamp, B. & Moyer, S. A. (1994). An amino-proximal domain of the L protein binds to the P protein in the measles virus RNA polymerase complex. *Virology* **205**, 540-545.

Hsu, V. P., Hossain, M. J., Parashar, U. D., Ali, M. M., Ksiazek, T. G., Kuzmin, I., Niezgoda, M., Rupprecht, C., Bresee, J. & Breiman, R. F. (2004). Nipah virus encephalitis reemergence, Bangladesh. *Emerg Infect Dis* **10**, 2082-2087.

Jack, P. J., Boyle, D. B., Eaton, B. T. & Wang, L. F. (2005). The complete genome sequence of J virus reveals a unique genome structure in the family Paramyxoviridae. *J Virol* **79**, 10690-10700.

Kang, D. C., Gopalkrishnan, R. V., Wu, Q., Jankowsky, E., Pyle, A. M. & Fisher, P. B. (2002). mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci U S A* **99**, 637-642.

Kato, A., Kiyotani, K., Sakai, Y., Yoshida, T. & Nagai, Y. (1997). The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. *Embo J* **16**, 578-587.

Kolakofsky, D., Roux, L., Garcin, D. & Ruigrok, R. W. (2005). Paramyxovirus mRNA editing, the "rule of six" and error catastrophe: a hypothesis. *J Gen Virol* **86**, 1869-1877.

Kumar, K. P., McBride, K. M., Weaver, B. K., Dingwall, C. & Reich, N. C. (2000). Regulated nuclear-cytoplasmic localization of interferon regulatory factor 3, a subunit of double-stranded RNA-activated factor 1. *Mol Cell Biol* **20**, 4159-4168.

Lamb, R. A. & Kolakofsky, D. (2001). Chapter 41: Paramyxoviridae: the viruses and their replication. In *Fields Virology*, pp. 1305-1340. Edited by H. P. K. DM. Philadelphia: Lippincott Williams & Wilkins.

Li, Z., Yu, M., Zhang, H., Magoffin, D. E., Jack, P. J., Hyatt, A., Wang, H. Y. & Wang, L. F. (2006). Beilong virus, a novel paramyxovirus with the largest genome of non-segmented negative-stranded RNA viruses. *Virology* **346**, 219-228.

Ludlow, L. E., Lo, M. K., Rodriguez, J. J., Rota, P. A. & Horvath, C. M. (2008). Henipavirus V protein association with Polo-like kinase reveals functional overlap with STAT1 binding and interferon evasion. *J Virol* **82**, 6259-6271.

Magoffin, D. E., Halpin, K., Rota, P. A. & Wang, L. F. (2007). Effects of single amino acid substitutions at the E residue in the conserved GDNE motif of the Nipah virus polymerase (L) protein. *Arch Virol* **152**, 827-832.

Mebatsion, T., de Vaan, L. T., de Haas, N., Romer-Oberdorfer, A. & Braber, M. (2003). Identification of a mutation in editing of defective Newcastle disease virus recombinants that modulates P-gene mRNA editing and restores virus replication and pathogenicity in chicken embryos. *J Virol* **77**, 9259-9265.

Middleton, D. J., Westbury, H. A., Morrissy, C. J., van der Heide, B. M., Russell, G. M., Braun, M. A. & Hyatt, A. D. (2002). Experimental Nipah virus infection in pigs and cats. *J Comp Pathol* **126**, 124-136.

Mohd Nor, M. N., Gan, C. H. & Ong, B. L. (2000). Nipah virus infection of pigs in peninsular Malaysia. *Rev Sci Tech* **19**, 160-165.

Murray, K., Rogers, R., Selvey, L., Selleck, P., Hyatt, A., Gould, A., Gleeson, L., Hooper, P. & Westbury, H. (1995a). A novel morbillivirus pneumonia of horses and its transmission to humans. *Emerg Infect Dis* **1**, 31-33.

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A., Gleeson, L., Westbury, H., Hiley, L., Selvey, L., Rodwell, B. & et al. (1995b). A morbillivirus that caused fatal disease in horses and humans. *Science* **268**, 94-97.

Nakatsu, Y., Takeda, M., Ohno, S., Koga, R. & Yanagi, Y. (2006). Translational inhibition and increased interferon induction in cells infected with C protein-deficient measles virus. *J Virol* **80**, 11861-11867.

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M. & Yanagi, Y. (2008). Measles virus circumvents the host interferon response by different actions of the C and V proteins. *J Virol* **82**, 8296-8306.

Niwa, H., Yamamura, K. & Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.

Park, M. S., Shaw, M. L., Munoz-Jordan, J., Cros, J. F., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A. & Basler, C. F. (2003). Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol* **77**, 1501-1511.

Parks, C. L., Witko, S. E., Kotash, C., Lin, S. L., Sidhu, M. S. & Udem, S. A. (2006). Role of V protein RNA binding in inhibition of measles virus minigenome replication. *Virology* **348**, 96-106.

Paterson, R. G., Leser, G. P., Shaughnessy, M. A. & Lamb, R. A. (1995). The paramyxovirus SV5 V protein binds two atoms of zinc and is a structural component of virions. *Virology* **208**, 121-131.

Paton, N. I., Leo, Y. S., Zaki, S. R., Auchus, A. P., Lee, K. E., Ling, A. E., Chew, S. K., Ang, B., Rollin, P. E., Umapathi, T., Sng, I., Lee, C. C., Lim, E. & Ksiazek, T. G. (1999). Outbreak of Nipah-virus infection among abattoir workers in Singapore. *Lancet* **354**, 1253-1256.

Pelet, T., Curran, J. & Kolakofsky, D. (1991). The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site. *EMBO J* **10**, 443-448.

Promed-mail (2006). Hendra virus, equine - Australia (NSW): susp.

Promed-mail (2007a). Hendra virus, human, equine - Australia In *Promed-mail*.

Promed-mail (2007b). Hendra virus, human, equine - Australia (QLD): RFI.

Promed-mail (2007c). Undiagnosed deaths - Bangladesh, India

Promed-mail (2008). 2 children die of suspected Nipah infection

Reynes, J. M., Counor, D., Ong, S., Faure, C., Seng, V., Molia, S., Walston, J., Georges-Courbot, M. C., Deubel, V. & Sarthou, J. L. (2005). Nipah virus in Lyle's flying foxes, Cambodia. *Emerg Infect Dis* **11**, 1042-1047.

Rodriguez, J. J., Cruz, C. D. & Horvath, C. M. (2004). Identification of the nuclear export signal and STAT-binding domains of the Nipah virus V protein reveals mechanisms underlying interferon evasion. *J Virol* **78**, 5358-5367.

Rodriguez, J. J. & Horvath, C. M. (2004). Host evasion by emerging paramyxoviruses: Hendra virus and Nipah virus v proteins inhibit interferon signaling. *Viral Immunol* **17**, 210-219.

Rodriguez, J. J., Parisien, J. P. & Horvath, C. M. (2002). Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. *J Virol* **76**, 11476-11483.

Rodriguez, J. J., Wang, L. F. & Horvath, C. M. (2003). Hendra virus V protein inhibits interferon signaling by preventing STAT1 and STAT2 nuclear accumulation. *J Virol* **77**, 11842-11845.

Roelke-Parker, M. E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S. J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H. & et al. (1996). A canine distemper virus epidemic in Serengeti lions (Panthera leo). *Nature* **379**, 441-445.

Rota, P. A., Bloom, A. E., Vanchiere, J. A. & Bellini, W. J. (1994). Evolution of the nucleoprotein and matrix genes of wild-type strains of measles virus isolated from recent epidemics. *Virology* **198**, 724-730.

Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P. & Basler, C. F. (2005). Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. *J Virol* **79**, 6078-6088.

Shaw, M. L., Garcia-Sastre, A., Palese, P. & Basler, C. F. (2004). Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. *J Virol* **78**, 5633-5641.

Shiell, B. J., Gardner, D. R., Crameri, G., Eaton, B. T. & Michalski, W. P. (2003). Sites of phosphorylation of P and V proteins from Hendra and Nipah viruses: newly emerged members of Paramyxoviridae. *Virus Res* **92**, 55-65.

Sleeman, K., Bankamp, B., Hummel, K. B., Lo, M. K., Bellini, W. J. & Rota, P. A. (2008). The C, V and W proteins of Nipah virus inhibit minigenome replication. *J Gen Virol* **89**, 1300-1308.

Smallwood, S. & Moyer, S. A. (2004). The L polymerase protein of parainfluenza virus 3 forms an oligomer and can interact with the heterologous Sendai virus L, P and C proteins. *Virology* **318**, 439-450.

Sweetman, D. A., Miskin, J. & Baron, M. D. (2001). Rinderpest virus C and V proteins interact with the major (L) component of the viral polymerase. *Virology* **281**, 193-204.

Takeuchi, K., Takeda, M., Miyajima, N., Ami, Y., Nagata, N., Suzaki, Y., Shahnewaz, J., Kadota, S. & Nagata, K. (2005). Stringent requirement for the C protein of wild-type measles virus for growth both in vitro and in macaques. *J Virol* **79**, 7838-7844.

Takeuchi, K., Tanabayashi, K., Hishiyama, M., Yamada, Y. K., Yamada, A. & Sugiura, A. (1990). Detection and characterization of mumps virus V protein. *Virology* **178**, 247-253.

Vanchiere, J. A., Bellini, W. J. & Moyer, S. A. (1995). Hypermutation of the phosphoprotein and altered mRNA editing in the hamster neurotropic strain of measles virus. *Virology* **207**, 555-561.

Wang, L. F., Michalski, W. P., Yu, M., Pritchard, L. I., Crameri, G., Shiell, B. & Eaton, B. T. (1998). A novel P/V/C gene in a new member of the Paramyxoviridae family, which causes lethal infection in humans, horses, and other animals. *J Virol* **72**, 1482-1490.

Wang, L. F., Yu, M., Hansson, E., Pritchard, L. I., Shiell, B., Michalski, W. P. & Eaton, B. T. (2000). The exceptionally large genome of Hendra virus: support for creation of a new genus within the family Paramyxoviridae. *J Virol* **74**, 9972-9979.

Wardrop, E. A. & Briedis, D. J. (1991). Characterization of V protein in measles virus-infected cells. *J Virol* **65**, 3421-3428.

WHO (2004). Nipah virus outbreak(s) in Bangladesh, January-April 2004. *Wkly Epidemiol Rec* **79**, 168-171.

Witko, S. E., Kotash, C., Sidhu, M. S., Udem, S. A. & Parks, C. L. (2006). Inhibition of measles virus minireplicon-encoded reporter gene expression by V protein. *Virology* **348**, 107-119.

Yamada, H., Hayata, S., Omata-Yamada, T., Taira, H., Mizumoto, K. & Iwasaki, K. (1990). Association of the Sendai virus C protein with nucleocapsids. *Arch Virol* **113**, 245-253.

Yob, J. M., Field, H., Rashdi, A. M., Morrissy, C., van der Heide, B., Rota, P., bin Adzhar, A., White, J., Daniels, P., Jamaluddin, A. & Ksiazek, T. (2001). Nipah virus infection in bats (order Chiroptera) in peninsular Malaysia. *Emerg Infect Dis* **7**, 439-441.

Yoneda, M., Guillaume, V., Ikeda, F., Sakuma, Y., Sato, H., Wild, T. F. & Kai, C. (2006). Establishment of a Nipah virus rescue system. *Proc Natl Acad Sci U S A* **103**, 16508-16513.

Yu, M., Hansson, E., Langedijk, J. P., Eaton, B. T. & Wang, L. F. (1998a). The attachment protein of Hendra virus has high structural similarity but limited primary sequence homology compared with viruses in the genus Paramyxovirus. *Virology* **251**, 227-233.

Yu, M., Hansson, E., Shiell, B., Michalski, W., Eaton, B. T. & Wang, L. F. (1998b). Sequence analysis of the Hendra virus nucleoprotein gene: comparison with other members of the subfamily Paramyxovirinae. *J Gen Virol* **79 (Pt 7)**, 1775-1780.

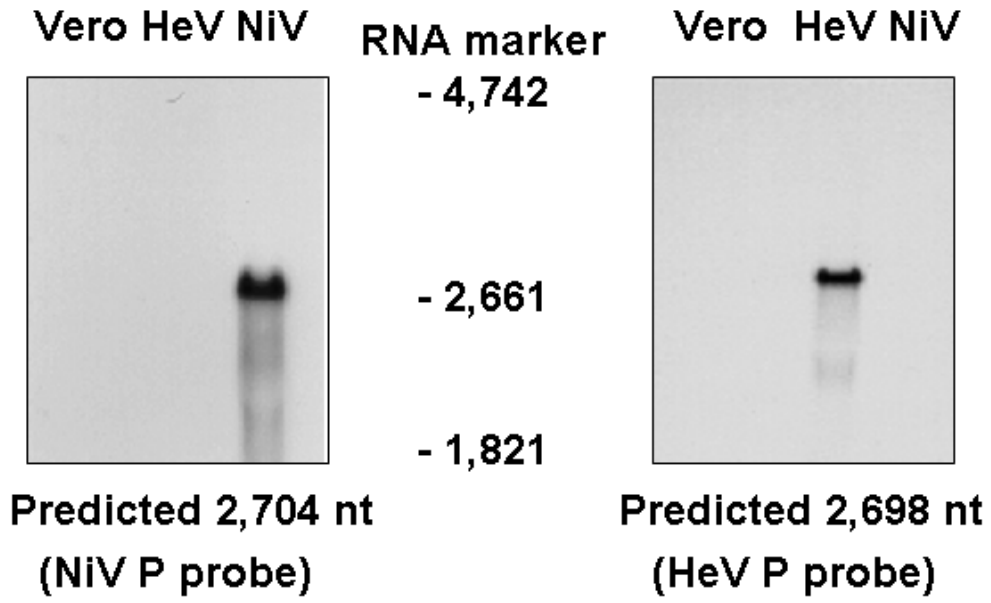
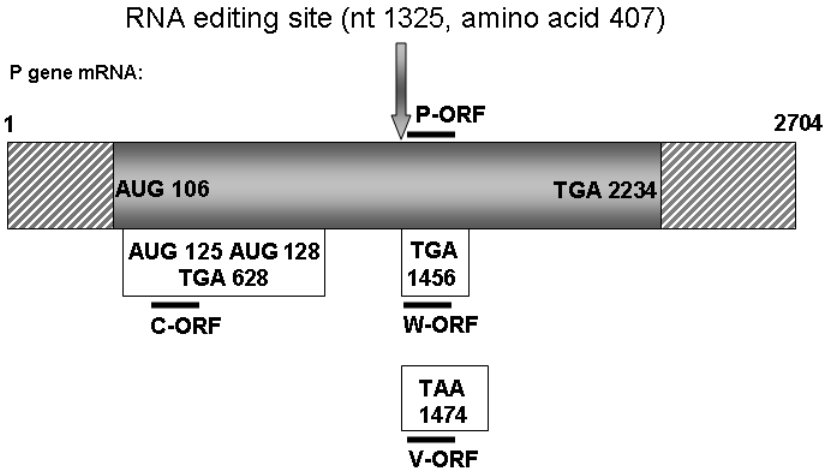


Figure 1. Northern blot analysis of P gene mRNA from NiV and HeV. Total RNA was isolated from mock, HeV, or NiV-M-infected Vero E6 cells. Five µg of RNA were electrophoresed with RNA markers (units in nucleotides, nt), transferred to nitrocellulose and probed with NiV or HeV-specific DIG-labeled probes. Both probes hybridized with an RNA moiety near the predicted size for each virus, 2704 nt for NiV and 2698 nt for HeV.

A



Peptide sequences & target location (amino acid positions)

P (411-450) KYPSAGTENVPGSKSGATRHVRGSPPYQEGKSVNAENVQL
V (411-450) EISICWDGKRAWVEEWCNPACSRITPLPRRQECQCGECPT
W (409-448) AQTRNIHLLGRKTCLGRRVVQPGMFEDHPPTKKARVSMRR
C (19-58) RHTDDQVFNNPASKIKQKPGKIFCSAPVENLNKLRGECLR

Figure 2A. The editing frequencies of the P gene transcripts of the henipaviruses. A diagram showing the multiple accessible ORFs in the NiV P gene due to mRNA editing (NiV V, W) and to usage of a downstream alternative ORF (NiV C). The targeted region of the anti P, anti C, anti V, and anti-W antipeptide sera are indicated by the black bar adjacent to each respective ORF, and their sequences and amino acid positions are indicated. The untranslated regions of the P gene are indicated by diagonal lines.

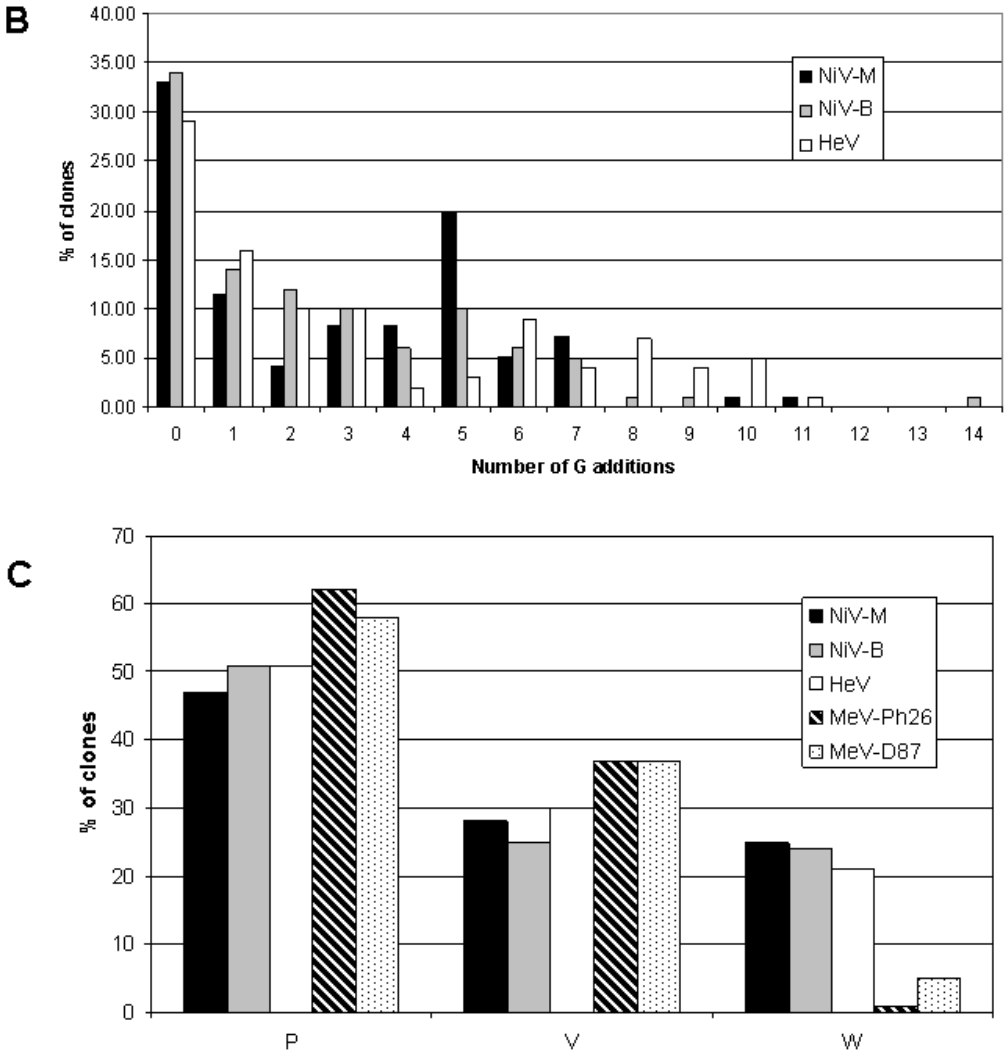


Figure 2B & 2C. The editing frequencies of the P gene transcripts of the henipaviruses. The percentage of plasmid clones containing 0-14 nontemplated G residues at the editing site for the henipaviruses, NiV-M, NiV-B, and HeV. C) The percentage of transcripts predicted to encode P, V, or W (see text) for the henipaviruses, NiV-M, NiV-B, and HeV, compared to two strains of measles virus, Philadelphia 26 (MeV-Ph-26) and Davis-87 (MeV-D87). Bars showing distribution of P gene transcripts of MeV were included for comparison and data were derived from previous reports (Bankamp *et al.*, 2008; Vanchiere *et al.*, 1995).

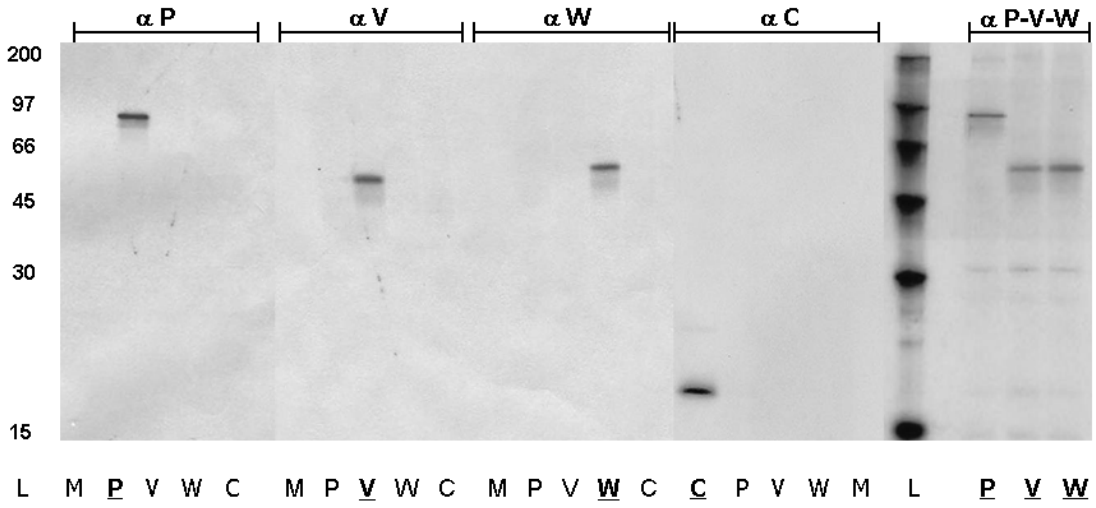


Figure 3. Expression of the P, C, V and W proteins of NiV in 293 cells and the specificity of the antipeptide antisera. 293 cells transfected with NiV P, C, V, W, or empty vector plasmid (M) were radiolabeled and lysates were immunoprecipitated with antipeptide antiserum against each of the indicated NiV proteins and electrophoresed on a 12% SDS PAGE gel. Letters at the bottom of the gel indicate the gene expressed in the cell lysate loaded into each well. The bolded and underlined letters identify the gene product corresponding to the specific antiserum used to perform the immunoprecipitations. Letters on the top of the gel with bracketed line segments indicate the specific antipeptide antiserum used to immunoprecipitate the cell lysate. In the 3 lanes on the far right of the gel, radiolabeled Vero cell lysates expressing P, V, and W were immunoprecipitated with the PVW peptide antiserum to confirm the electrophoretic mobililty of each protein.

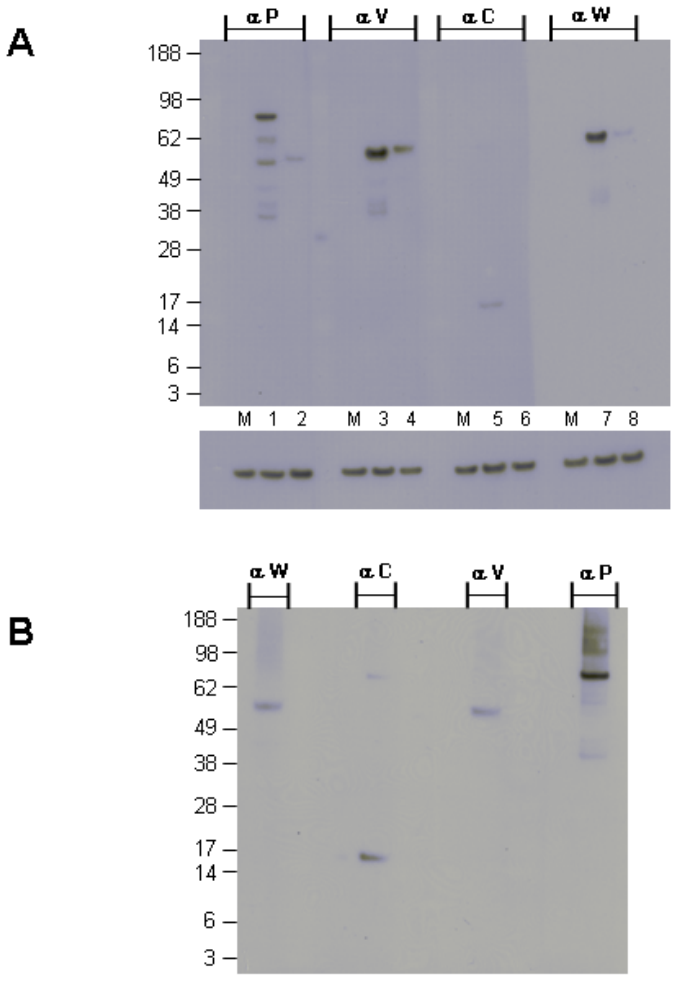


Figure 4. Expression of the P, C, V and W proteins of NiV in infected Vero cells and in purified virions. NiV infected cells and purified NiV virion lysates were examined by Western blot with antipeptide mouse antisera. A) Vero cells infected with NiV and HeV (MOI= 1) were lysed after 36 hours, and denatured in 2% SDS. Six µl of infected cell lysate were run in each well of a 4-12% gradient SDS PAGE gel. The antipeptide antiserum used to probe each section of the blot is indicated by letters at the top of the gel. Lanes 1, 3, 5, and 7 are NiV-infected lysates, and lanes 2, 4, 6 and 8 are HeV-infected lysates. 'M' indicates mock-infected lysates. Well-loading control blots with antibody to cellular β–actin are shown below. B) Two µl/well of sucrose-gradient purified NiV was used to perform Western blots as described in A.

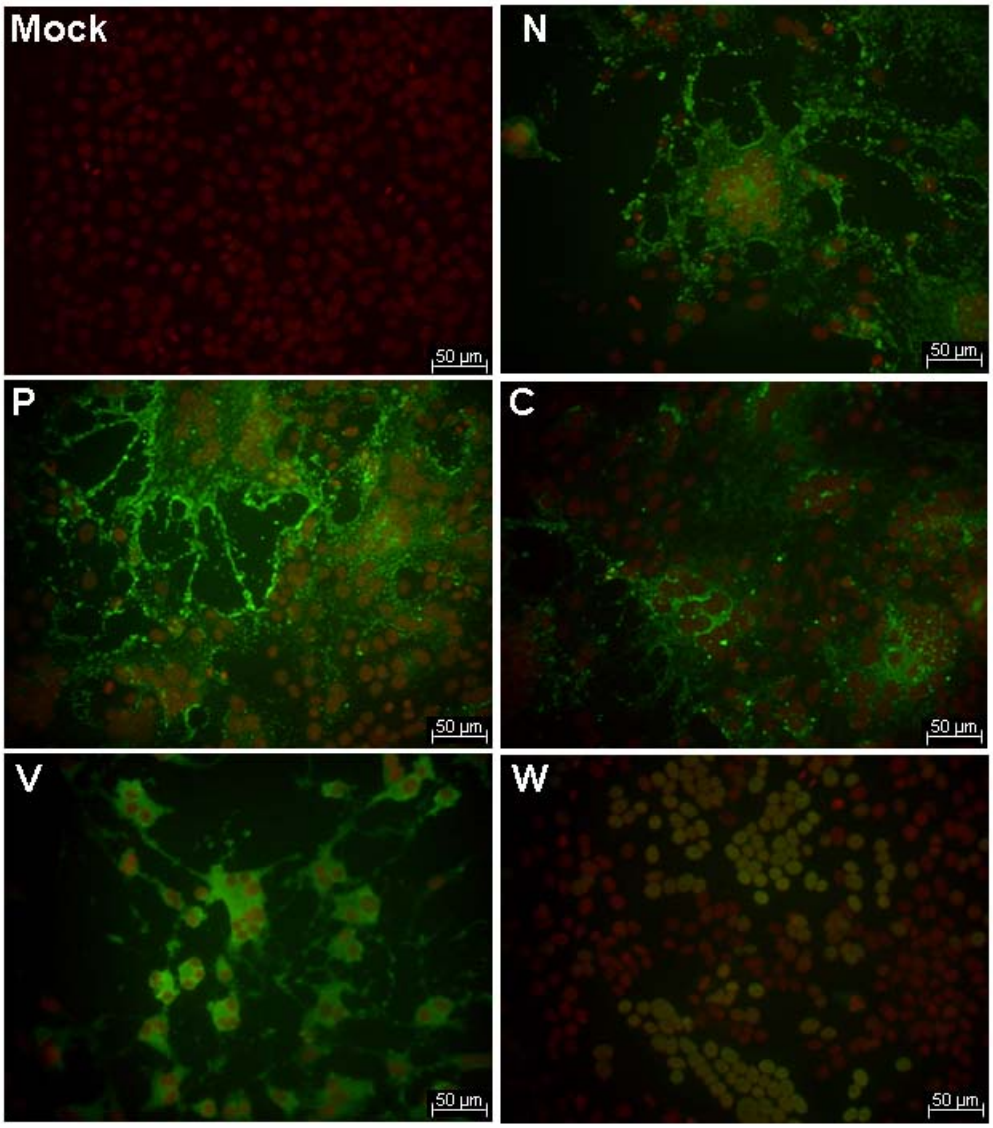


Figure 5. Cellular distributions of P V, W, and C proteins of NiV. Vero cells were infected with NiV at an MOI of 0.01 for 24 hours, fixed in methanol, and probed with specific antipeptide antisera against NiV N, P, C, V, or W. 'M' is a representative picture of mock-treated cells. A secondary anti-mouse/rabbit IgG conjugated with Alexafluor-488 was used for detection (green), and DAPI was used to stain the nuclei (shown in red for contrast).

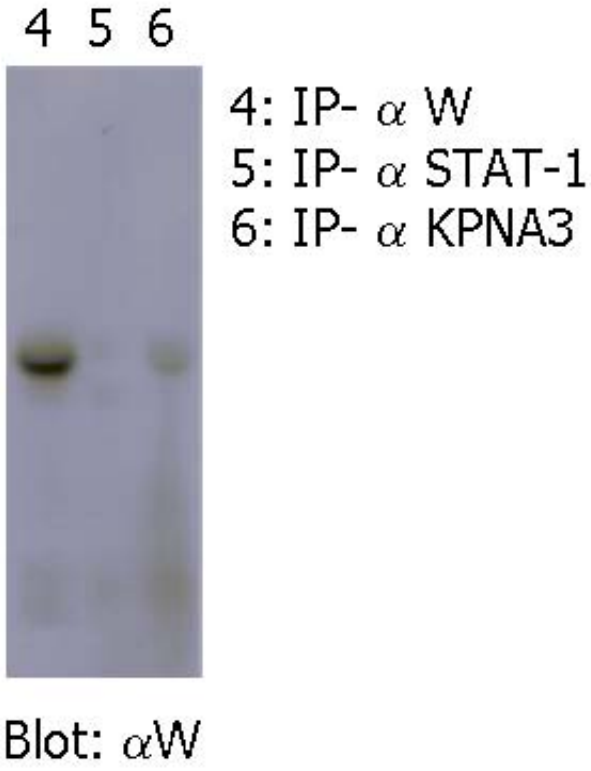


Figure 6. Interaction between NiV W and KPNA3. NiV infected cell lysate samples were immunoprecipitated (IP) using the indicated antibody for each well, electrophoresed on a 4-12% SDS gel, and transferred onto a PVDF membrane. Western blot was performed to determine the presence of W.

Chapter 3

Characterization of the growth kinetics and the innate immune response against Nipah virus in endothelial cells and neurons

Michael K. Lo[1,2] , David Miller[3], Mohammad Aljofan[4], Bruce A. Mungall[4], Pierre E. Rollin[3], William J. Bellini[1], and Paul A. Rota[1]

[1]Measles, Mumps, Rubella, and Herpesvirus Laboratory Branch, Centers for Disease Control and Prevention, Mail-stop C-22, 1600 Clifton Road, Atlanta, Georgia 30333

[2]Emory University, Laney Graduate School, Graduate Division of Biological and Biomedical Sciences, Immunology and Molecular Pathogenesis Program, 1462 Clifton Road Suite 314, Atlanta, GA 30322

[3]Special Pathogens Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, Georgia 30333

[4]Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organization, 5 Portarlington Road, Geelong, Victoria 3220, Australia

Contribution of other authors to this body of work: The immunolabeling assay was performed by M.A., and all other live Nipah virus infections were performed by D.M.

Abstract

Nipah virus (NiV) is a highly pathogenic paramyxovirus which causes fatal encephalitis in up to 75% of infected human cases. It has been demonstrated that endothelial cells and neurons are important cellular targets in the pathogenesis of this disease. In spite of several elegant studies showing that individual NiV P gene products can antagonize the innate antiviral response in several cell lines, it is not known whether live NiV infection of physiologically relevant targets mirrors the results seen in those plasmid expression studies. Furthermore, there is a paucity of data in regards to the molecular mechanisms which explain how the extensive vasculitis is perpetuated in human infections. In this study, we characterized the growth kinetics and the innate immune responses of various primary endothelial cells and a neuronal cell line. We demonstrated that various NiV infected endothelial cells generated a functionally robust IFN-β response, which correlated with a differential localization of the NiV W protein compared with infected neuronal cells in which there was no antiviral response. We also determined that NiV infection of endothelial cells induced a significant increase of inflammatory chemokines secreted into the cellular supernatant, and that these supernatants induced a corresponding increase in monocyte and T-lymphocyte chemotaxis.

Introduction

Nipah virus (NiV) is a highly pathogenic member of the family *Paramyxoviridae*, in the subfamily *Paramyxovirinae*, in which it along with Hendra virus makes up the *Henipavirus* genus (Lo & Rota, 2008). A common characteristic shared by paramyxoviruses is their ability to generate multiple proteins from a single gene by mRNA editing and usage of alternative open reading frames (Curran & Kolakofsky, 1990; Giorgi *et al.*, 1983; Kolakofsky *et al.*, 2005; Liston & Briedis, 1995). These additional gene products typically have the ability to antagonize certain antiviral induction and/or signaling pathways (Gotoh *et al.*, 2002; Horvath, 2004). Plasmid expression of the NiV C, V, and W proteins indicated their varying abilities to antagonize the innate antiviral response in avian and human cell lines. The NiV C, V, and W proteins were able to rescue the replication of an interferon (IFN) sensitive Newcastle Disease virus (Park *et al.*, 2003). The N-terminal region shared by the NiV P, V, and W proteins has been shown to bind to signal transduction activator of transcription (STAT)-1, thus preventing its phosphorylation (Rodriguez *et al.*, 2004; Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). In addition, it was shown that the nuclear localization of the NiV W protein enhanced its ability to antagonize the activation of interferon regulatory factor (IRF) 3 (Shaw *et al.*, 2005). However, it was not until more recently that these proteins were characterized in the context of a viral infection. We have shown that the phosphoprotein (P) gene mRNA of NiV is edited at a high frequency to yield the NiV V and W proteins, and that an alternative reading frame in the P gene is indeed utilized to generate the C protein. We have also demonstrated the expression and distinct subcellular localizations of NiV C (punctuate cytoplasmic), V (homogenous

cytoplasmic), and W (nuclear) proteins in Vero cells, and detected the presence of these proteins in purified NiV virions (Lo *et al.*, 2009). In accordance with results from a study indicating an interaction between plasmid expressed NiV W and karyopherin α3/4 (KPNA3/4), we were able to detect this protein-protein interaction in NiV infected cells (Lo *et al.*, 2009; Shaw *et al.*, 2005). More recently, a reverse genetic study indicated a crucial role of the NiV C protein in viral replication, and showed that phosphorylated STAT-1 was precluded from moving into the nuclei of wild-type NiV infected Vero E6 cells while a STAT-binding mutant virus allowed for its translocation (Ciancanelli *et al.*, 2009). The results of these studies utilizing live NiV brings up the question of whether NiV infection of physiologically relevant target cells produces similar results as seen in these tumorized, non-IFN producing cell lines.

NiV causes severe encephalitis in humans, characterized by systemic vasculitis and necrosis particularly in the central nervous system (CNS). Extensive infection of neurons, endothelial cells, and smooth muscle cells of blood vessels is characteristic of human NiV infections (Wong *et al.*, 2002). The molecular basis of NiV pathogenesis is still unclear. The inflammatory cellular infiltrate found in the central nervous system (CNS) during NiV encephalitis include neutrophils, macrophages, lymphocytes, and reactive microglia (Wong et al., 2002). The recruitment of these cells indicates the possible action of small molecule messengers and chemoattractants known as cytokines and chemokines. Some inflammatory cytokines found in infectious CNS pathology include TNFα and IL-6, while some chemokines include IL-8 (CXCL8), RANTES (CCL5), MIP-1α (CCL3), MCP-1 (CCL2), and IP-10 (CXCL10) (Glabinski & Ransohoff, 1999). The cellular sources of these secreted cytokines are varied, as cells of

the CNS as well as the inflammatory infiltrate have the ability to produce and secrete these molecules (Griffin, 1997; Owens et al., 2005). These cytokines and chemokines have also been correlated to pulmonary inflammation due to mycobacterium and Respiratory Syncytial virus (RSV) infection (Kunkel *et al.*, 2002; Pace *et al.*, 1999; Penido *et al.*, 2003). In order to address the physiological relevance of previous studies performed in Vero cells, we characterized and compared the growth kinetics of NiV in several primary human endothelial cells and a human neuroblastoma cell line. Our findings indicate a significant difference in viral replication rate between neurons and endothelial cells. This difference corresponded to the ability of endothelial cells to generate an IFN-β response, which correlated with a differential localization of NiV W between endothelial cells and neurons. We also demonstrated a significant increase of inflammatory chemokines present in supernatants of NiV infected cells, and showed that increases in these particular chemokines correlated with increased induction of functional monocyte and T-lymphocyte chemotaxis.

Results

The rate of NiV replication is significantly higher in neuronal cells than in endothelial cells. In order to measure and compare the infectivity of human endothelial and neuronal cells by NiV, a chemiluminescent immunolabeling assay was performed in which levels of NiV nucleoprotein (N) detected served as an indicator of viral infection (Aljofan *et al.*, 2008). Significant levels of NiV antigen could not be detected in the EA926.hy HUVEC-A549 fusion hybrid cells, which served as a negative control. At 16 hours post-infection, we found that while all the human endothelial cells were infected with NiV, the levels of NiV N detected in the M17 neuronal cells were at least twice the levels found in any of the endothelial cells used in this assay (Figure 1). To determine whether this disparity in NiV N protein expression levels was due to a difference in viral growth kinetics between neuronal cell lines, a single-step growth curve was performed for HUVEC, LYMEC, HULEC, HBEC, and M17 cells (Figure 2). At 16 hours post-infection (MOI=1), M17 cells had already generated significant amounts of infectious virus particles (~10^7 $TCID_{50}$/mL), which indicated a high rate of viral replication. Peak viral titers were achieved in infected M17 cells by 24 hours post-infection; the drop in viral titer at 32 hours post-infection was likely due to both the lack of cells remaining to infect as well as virus degradation. In contrast, the endothelial cells infected with NiV, the number of virus particles produced was barely above the limit of detection by 16 hours post-infection (Figure 2). Given the relative similarity in NiV growth kinetics among the endothelial cells compared to that of neuronal cells, the different levels of NiV antigen detected between HULEC, HUVEC, and HBEC at 16 hours post-infection (Figure 1) could indicate a difference in susceptibility to NiV infection, such as a

difference in the abundance of host cell receptors Ephrin B2/B3. To ensure that a majority of endothelial cells were being infected, we repeated the growth curve experiment in HUVECs but increased the MOI to 2. By increasing the input virus, there was a substantial increase of almost 1.5 logs in viral titer at 16 hours post-infection (Figure 2B). Nevertheless, the rate of replication still did not reach that of NiV in M17 cells, which yielded 100-fold more virus particles at the same time point. Notably, in spite of the significant increase in titer earlier in HUVEC infection provided by increasing the MOI, there was only a modest (3-fold) difference in titer by 48 hours post-infection. The fact that the HUVECs supported NiV replication to the same extent as the M17 cells but reached that titer later in infection (64 hours) suggested that innate cellular response mechanisms in endothelial cells may potentially limit early viral replication (Figure 2B).

NiV infected endothelial cells produce functionally significant levels of IFN-β. In order to determine the presence or absence of an innate antiviral response to NiV infection, we performed IFN-β ELISAs on gamma-irradiated supernatants collected from infected cells. While we did not detect any significant levels of IFN-β from NiV infected M17 cell supernatants (data not shown), we detected the presence of IFN-β from endothelial cell supernatants at levels above baseline (mock-infected cells) as early as 16 hours post-infection (Figure 3A, HULEC). The HULECs produced the highest levels of IFN-β in response to NiV infection. The LYMECs also generated IFN-β in response to NiV infection with similar kinetics, but produced lower absolute levels of IFN-β compared to HULECs. The HUVECs had a slightly delayed IFN-β response compared

with the HULECs and LYMECs, and also produced 5-fold lower levels of IFN-β than the other two endothelial cell types (Figure 3C). For positive controls, each endothelial cell type was treated with 50 μg/mL of poly-I:C for 24 hours before the supernatant was harvested to be assayed for IFN-β. The absolute capacity to produce IFN-β seemed to depend upon endothelial cell type. Both the lung-derived HULECs and LYMECs produced ~ 20-25 IU/mL of IFN-β when stimulated with poly I:C, while HUVECs only produced ~ 4 IU/mL of IFN-β. We observed that the induction of IFN-β in the endothelial cells required viral replication, as UV-inactivated virus failed to induce the endothelial cells to produce significant levels of IFN-β (Figure 3).

To determine whether the IFN-β produced from NiV infected endothelial cells was functional, we performed an Encephalomyocarditis virus cytopathic effect (EMCV CPE) assay. Pre-treatment of A549 human lung airway epithelial cells with supernatants taken from NiV infected HULECs at 16, 24, 48, and 64 hours provided significant protection of the cells from EMCV-induced CPE (Figure 4), while supernatants taken from NiV infected LYMECs at 24, 48, and 64 hours provided substantial protection, with the 24 hour supernatant providing 2-fold less protection than the corresponding time point from HULEC supernatants. In accordance with the comparatively lower levels of IFN-β produced by HUVECs as observed in the IFN-β ELISA, pretreatment of A549 cells with NiV infected HUVEC supernatants provided some protection from EMCV CPE, but the level of protection was not as substantial as those provided by NiV infected HULEC and LYMEC supernatants (Figure 4).

Cell-type specific subcellular localization of NiV W. We have shown previously that the accessory proteins (NiV C, V, and W proteins) derived from the NiV P gene have distinct subcellular localizations in NiV infected Vero cells (Lo *et al.*, 2009). We performed immunofluorescence assays on the NiV infected neuronal and endothelial cells to determine whether these proteins localized to similar compartments as they appear in Vero cells. Interestingly, we found that while the NiV W localized to the nucleus of M17 cells as it did in Vero cells, it was primarily found in the cytoplasm of all endothelial cell types that we infected with NiV (Figure 5). In the context of plasmid expression, the NiV V and W proteins have been shown to have antagonistic activities for IFN-β promoter activation by Sendai virus infection. The nuclear localization of plasmid-expressed NiV W allows it to block both TLR-3 and virus-induced IFN-β induction pathways (Shaw *et al.*, 2005). Our finding that the majority of NiV W protein produced during infection of endothelial cells is excluded from the nucleus indicates the presence of additional antiviral mechanisms inherent to primary endothelial cells that allow for the production of IFN-β during NiV infection.

NiV infection induces production of inflammatory chemokines and promotes monocyte and T-lymphocyte chemotaxis. One of the main pathological findings from human cases of NiV is vasculitis characterized by focal and transmural mixed inflammatory infiltrates consisting of neutrophils, macrophages, lymphocytes, and microglia (Wong *et al.*, 2002). Since endothelial cells are one of the prime targets of NiV infection, we wanted to determine whether endothelial cells could serve as a source of inflammatory cytokines and chemokines that would contribute to the manifestation of

vasculitis seen in human disease. Luminex multiplex bead array assays were performed to detect the presence of inflammatory cytokines and chemokines in NiV infected HULEC and LYMEC supernatants. In spite of a gradual increase in levels of MCP-1 and IL-8 in mock treated endothelial cell supernatants due to basal levels of expression of both chemokines that accumulates over time, we detected significant increases in levels of IL-8, MCP-1, IP-10, and IL-6 (IL-6 upregulation only in HULECs - data not shown) consistently by 48 hours post infection (Figure 6A, C, E, G; 7A & C).. We demonstrated that viral replication was required in order to induce upregulated levels of these cytokines, as UV inactivated virus stocks did not significantly increase levels of these chemokines at any of the corresponding time points in which we harvested supernatants (Figures 6A, C, E, G; 7A, C). The substantial increases (from 4 fold to 60-fold) we observed in mRNA transcription of these genes over baseline levels generally corresponded to the time points in which we saw increased levels of these proteins in the supernatants, (Figures 6B, D, F, H; 7B & D). In order to determine any functional significance correlated with these upregulated chemokines, we performed chemotaxis assays using the THP-1 monocyte and the JE-6.1 T lymphocyte cell lines. We observed that the supernatants taken from NiV infected HULEC and LYMECs at 48 and 64 hours post-infection induced a significant increase in both monocyte and lymphocyte chemotaxis over mock supernatants (Figure 6I, J, 7C, D). Supernatants taken from poly-I:C stimulated endothelial cells consistently induced high levels of chemotaxis, and were used as the positive control representing maximum induction of chemotaxis.

Discussion

This is the first report characterizing the innate antiviral responses of primary endothelial cells against NiV infection. Despite previous reports of the ability of NiV V and W proteins to block IFN-β production via TLR-3 and mda-5 mediated signaling (Parisien *et al.*, 2009; Shaw *et al.*, 2005), we have shown that NiV infected primary human endothelial cells can generate a functional IFN-β response. Surprisingly, we found that the majority of NiV W protein detected by immunofluorescence assays of NiV infected endothelial cells localized to the cytoplasm, contrary to its localization in infected M17 neuronal cells shown in this study, as well as in infected Vero cells and plasmid-transfected HeLa cells shown in prior studies (Lo *et al.*, 2009; Shaw *et al.*, 2005). Since nuclear localization of plasmid-expressed NiV W was shown to be required to block TLR-3 signaling by over expression of TRIF, it is possible that TLR-3 signaling is the means by which primary endothelial cells generate the observed IFN-β response to NiV infection. Another possibility regarding the source of IFN-β production is via the RIG-I RNA helicase pathway. RIG-I has been shown to be responsible for inducing cytokine responses to the stem loop structured leader of measles virus, as well as to Sendai virus infection (Melchjorsen *et al.*, 2005; Plumet *et al.*, 2007; Shingai *et al.*, 2007). Parainfluenza simian virus 5 (PIV5) has also been shown to organize its genomic promoter sequence in a way to limit detection by RIG-I (Manuse & Parks, 2009). While many paramyxovirus V proteins (including that of NiV) have been shown to bind and inhibit the antiviral signaling activity of mda-5, they have not had the same effect on RIG-I (Childs *et al.*, 2007; Childs *et al.*, 2009; Parisien *et al.*, 2009). It is possible that in spite of the ability of NiV infected endothelial cells to mount an IFN-β response as early

as 16 hours post infection, NiV is able to overcome the effects of IFN-β signaling, because we see a continual increase in virus titer in spite of IFN-β production (Figures 2 & 3). It is worth investigating the cellular sublocalization of phosphorylated/unphosphorylated Interferon Regulatory Factors 3 and 7 (IRF-3 & IRF-7) in the context of NiV infection of endothelial cells to determine possible mechanisms that provide avenues for IFN-β production. The results obtained from the immunofluorescence assays of NiV W protein in this study highlight the benefits of utilizing primary endothelial cells in attempting to study the pathogenesis of NiV infection, as they significantly differ from infections performed in both IFN-incompetent and competent cell lines (Vero, Vero E6, 293T) (Ciancanelli *et al.*, 2009; Lo *et al.*, 2009).

While the macroscopic pathological features of NiV infection in humans has been well described (Wong *et al.*, 2002), the molecular mechanisms underlying the pathogenesis of NiV infection remain largely undefined. Given the pronounced vasculitis observed in the CNS and lungs of human cases, we thought that measuring the presence of inflammatory chemokines secreted by primary targets of NiV infection would identify possible mechanisms by which the observed vasculitis is induced. Chemokines are a diverse family of low molecular weight proteins that play a major role in guiding the migration of cells divided into 4 subfamilies based on structure and function. Two major subfamilies include the CXC and CC chemokines. CXC chemokines are split into two types: those that contain a glutamate-leucine-arginine (ELR) motif, and those that do not (non-ELR). CXC chemokines that have the ELR motif (i.e., IL-8/CXCL8) primarily attract neutrophils and are associated with bacterial infections, but have limited effects on T cells and monocytes. Non-ELR CXC chemokines (i.e., IP-10/CXCL10) tend to attract

activated T cells and NK cells, and are typically associated with antiviral activities (Mahalingam *et al.*, 1999). On the other hand, CC chemokines (i.e., MCP-1/CCL2) attract T cells, monocytes, and macrophages, but not neutrophils (Lane *et al.*, 2006). We have shown that NiV infection of human lung and lung lymphatic endothelial cells indeed results in the upregulation of inflammatory chemokines MCP-1, IL-8, and IP-10 which may contribute to the induction of both monocyte and T-lymphocyte chemotaxis. It has been demonstrated in clinical settings that epyema and parapneumonic effusions are associated with upregulated levels of MCP-1 and IL-8 that functionally induce chemotaxis (Antony *et al.*, 1993). A cDNA array study of respiratory syncytial virus (RSV) infection of human airway epithelial cells indicated an increase in IL-8 and MCP-1, which correlates with observed peribronchial mononuclear infiltrate, with eosinophilic and basophilic degranulation in human infections (Zhang *et al.*, 2001). Alveolar hemorrhage, pulmonary edema, and aspiration pneumonia were seen in the initial NiV outbreak, as well as in subsequent Bangladesh outbreaks, in which an increase in the rate of severe respiratory distress was reported (Goh *et al.*, 2000; Hossain *et al.*, 2008; Lee *et al.*, 1999; Paton *et al.*, 1999; Wong *et al.*, 2002). Importantly, MCP-1, IL-8, and IP-10 have been shown to be upregulated in both experimental infectious and non-infectious CNS pathology in monkeys, mice and rats (Glabinski & Ransohoff, 1999). All three chemokines have been shown to play a role in lymphocyte trafficking in CNS diseases (Klein, 2004; Loetscher *et al.*, 1994; Pace *et al.*, 1999; Penido *et al.*, 2003). MCP-1 has also been shown to regulate permeability of the blood-brain-barrier through altering the arrangement of endothelial tight junction proteins (Stamatovic *et al.*, 2005). Clinical studies of patients infected with Japanese encephalitis virus showed that infection is

correlated with elevated levels of IL-8 in the cerebrospinal fluid (CSF) (Singh *et al.*, 2000). Studies of West Nile virus infection in the mouse model indicate that IP-10 is induced in neurons and promotes T-cell trafficking (Klein *et al.*, 2005; Zhang *et al.*, 2008). CXCR3, the receptor for IP-10, is one of several prominent chemokine receptors expressed in the CNS at various stages of mouse hepatitis virus (MHV)-induced encephalomyelitis (Lane *et al.*, 2006). NDV has also been shown to induce the mouse homolog of IP-10 (Crg-2) in glial cells and astrocytes, while measles virus was demonstrated to induce IP-10 in glioblastoma cells (Cheng *et al.*, 1998; Fisher *et al.*, 1995; Nazar *et al.*, 1997; Vanguri & Farber, 1994). Since IP-10 by its name (interferon induced inflammatory protein) is widely known to be induced by IFN-γ, our detection of increased levels of IP-10 in NiV infected endothelial cells was somewhat unexpected, as IFN-γ is primarily produced by activated T cells. There are however other signaling pathways that do not require IFN-γ which can contribute to IP-10 expression. The HIV Tat protein has been shown to induce IP-10 via the p38 MAP kinase pathway and to induce IL-8 and MCP-1 via the ERK1/2 pathway (Kutsch *et al.*, 2000). The MAP kinase and ERK1/2 pathways have not yet been examined as potential players in the pathogenesis of NiV infection. The finding that RIG-I mediated induction of IFN-β and activation of dendritic cells requires an intact p38 MAP kinase pathway emphasizes the need for the investigation of these respective signaling pathways (Mikkelsen *et al.*, 2009). Another important if not overlooked area of study is the impact of NiV induced chemokines on the action of matrix metalloproteinases in the CNS (Khuth *et al.*, 2001; Owens *et al.*, 2005). It is notable that even though we have correlated upregulated inflammatory chemokine levels with increased monocyte and T-lymphocyte chemotaxis,

incubating NiV infected supernatants with antibodies against IL-8 and MCP-1 concomitantly and against IP-10 individually did not ablate the increased levels of chemotaxis (data not shown). This indicates that there are other inflammatory chemokines involved that were not addressed in this study. Future studies will focus on identification of specific chemokines crucial to inducing increased levels of monocyte and T-lymphocyte chemotaxis.

In summary, we have characterized the replication rates of NiV infection in neuronal and endothelial cells, detected endothelial antiviral responses which correlated with an unexpected localization of the NiV W protein, and have characterized the innate inflammatory response of endothelial cells to NiV infection. Our findings suggest that the induction of pro-inflammatory chemokines in NiV infected primary endothelial cells *in vitro* is consistent with the observed prominent vasculitis seen in human cases of NiV infection, and provide initial molecular insights into the details that explain these observations seen in human disease.

Materials and Methods

Cells and Viruses. Primary human umbilical cord vein endothelial cells (HUVEC), human lymphatic lung microvascular endothelial cells (LYMEC), and human lung microvascular endothelial cells (HULEC) were obtained from Lonza. HUVECs were maintained according to manufacturer's instructions in endothelial basal media (EBM) supplemented with aliquots of bovine brain extract with heparin, human epidermal growth factor, hydrocortisone, fetal bovine serum (FBS) (up to 2 % final volume) and antibiotics Amphotericin B and Gentamicin, all of which were supplied by Lonza. LYMECs and HULECs were maintained in EBM-2 (Lonza) supplemented with aliquots of human epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor, insulin-like growth factor, FBS (up to 5% final volume), hydrocortisone, and antibiotics Amphotericin B and Gentamicin all provided by Lonza. Endothelial cells obtained from Lonza were used within the first 5 passages, and were cultured in flasks pre-treated with 0.1% gelatin (Sigma). An immortalized human brain endothelial cell line (HBEC) was obtained from the Cell Culture Development Team at CDC, and was used within passage number 8-15. HBECs and EA926.hy cells (hybrid fusion of HUVEC and A549 cells) were maintained in M199 medium supplemented with 15% fetal bovine serum (FBS) (GIBCO-BRL Life Technologies, Inc., Frederick, MD), 16 U/ml heparin (ESI Pharmaceuticals, Cherry Hill, NJ), 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. BE(2)-M17 human neuroblastoma cells (M17) were obtained from ATCC and maintained in OPTI-MEM supplemented with 5% FBS. THP-1 monocytic leukemia cells and Jurkat JE-6.1 cells (ATCC TB-152) were maintained in RPMI 1640 with 10% FBS, 100U/ml penicillin and

100U/ml streptomycin. Vero and A549 cells were maintained in DMEM with 10% FBS, 100U/ml penicillin and 100U/ml streptomycin. All cell lines were cultured at 37°C with 5% CO_2. All work with live NiV was performed under biosafety level 4 conditions in the Maximum Containment Laboratory (MCL) at the Centers for Disease Control and Prevention or at the Australian Animal Health Laboratory (AAHL). The NiV stock used in these experiments were from an isolate from the Malaysian outbreak in 1999 (Chua *et al.*, 2000), passed four times on Vero E6 cells. Any potentially infectious material removed from the MCL was either sterilized with 5 x 10^6 rad in a Cobalt-60 gamma cell irradiator or chemically denatured with TRIZOL reagent (Invitrogen). Encephalomyocarditis virus (EMCV) was propagated in HeLa cells and had a titer of ~ 4.95 X 10^5 $TCID_{50}$/mL.

NiV Immunolabeling assay. Assays were performed as previously described (Aljofan *et al.*, 2008). Briefly, 96-well white plates of NiV infected endothelial and neuronal cells fixed with 100% cold methanol 16 hours post-infection were washed 3 times with Phosphate Buffered Saline containing 0.05% Tween-20 (PBS-T). Plates then were protein blocked with 100 ml of 2% skim milk in PBS-T and incubated at 37^{o}C for 30 minutes. After protein blocking, plates were washed 3 times with PBS-T, followed by incubation with 100 μL anti-NiV antibody (prepared by AAHL's Bioreagents Development Group) diluted 1:1000 in PBS-T containing 2% skim milk for 30 minutes at 37^{o}C and then washed 3 times with PBS-T. Plates were incubated with 1% H_2O_2 (Sigma) for 15 minutes at room temperature then washed with PBS-T 3 times. Anti-rabbit HRP conjugated antibody 100 μL (Sigma) diluted 1:2,000 in PBS-T containing 2% skim milk,

were added to each well and plates incubated at 37^{o}C for 30 minutes. Plates were washed 3 times with PBS-T and 100 ul aliquots of Chemiluminescent Peroxidase Substrate-3 (CPS-3, Sigma) diluted 1:10 in Chemiluminescent assay buffer (20 mM Tris-HCl, 1 mM $MgCl_2$, pH 9.6) were added to all wells. Plates were incubated at room temperature for approximately 15 minutes, and then read using a Luminoskan Ascent luminometer (Thermo) using 100msec integration per well.

Immunofluorescence Assay- ~ 5 X 10^4 neuronal or endothelial cells were seeded onto 8-chamber glass slides pre-treated with 0.1% gelatin (for endothelial cells), and infected with NiV at multiplicity of infection (MOI) of 0.1 (for M17) to 1 (endothelial cells). After 16-48 hours, slides were fixed with cold methanol for 15 minutes, and upon removal of the methanol, were allowed to dry at room temperature before either being stored at -20°C or blocked with blocking buffer (PBS, 0.1% Triton X-100, 10% goat serum) for 5-10 minutes. Primary antibodies generated against NiV W (Lo *et al.*, 2009) was diluted in blocking buffer at a dilution of 1:500, then added to the chamber slides which were incubated at room temperature for 45 minutes. After 3 washes with blocking buffer, slides were incubated with a secondary goat anti-mouse antibody conjugated with FITC diluted 1:1000 in blocking buffer, along with a 1:1000 dilution of DAPI to stain the nuclei. Slides were viewed on an AxioScope microscope, and AxioVision version 4.7 was used to capture images (Zeiss).

One-step growth curve/Median tissue culture infectious dose ($TCID_{50}$) analysis- Briefly, 1.25 X 10^5 to 4.5 X 10^5 cells were seeded on 6-well plates and infected for one hour at a

multiplicity of infection (MOI) of 1 (for M17) or 2 (for endothelial cells). Inoculum was removed and replaced with 1.5-2 mL of fresh media per well. Supernatants of infected cells were collected at 5, 8, 16, 24, 32, 48, and 64 hours post-infection and were serially diluted ten-fold to infect ~ 3-4 X 10^4 Vero cells in a 96-well plate to determine the $TCID_{50}$/mL, which was calculated using the Spearman-Karber method (Ballew, 1986). To generate IFN-β control supernatants, endothelial cells were treated with 50 mg/mL of polyinosinic:polycytidylic acid (poly I:C) for 24 hours before the supernatants were harvested and gamma-irradiated as mentioned above.

Luminex® multiplex cytokine bead assay- 100 μL of gamma-irradiated supernatant per sample was subjected to a Luminex multiplex cytokine bead assay according to manufacturer's protocols (Invitrogen). Briefly, supernatants were incubated for 2 hours in a 96-well filter plate with a mixture of beads conjugated with antibodies specific for Interleukin 6 (IL-6), (IL-8/CXCL8), IL-1β, tumor necrosis factor alpha (TNF-α), inflammatory protein 10 (IP-10/CXCL 10), and monocyte chemoattractant protein 1 (MCP-1/CCL2). After two washes, the beads were incubated for 1 hour with biotinylated detector antibodies specific for different epitopes of aforementioned cytokines and chemokines. After another two washes, the beads were incubated with streptavidin conjugated with phycoerythrin (PE) for 30 minutes. After 3 washes, the beads were resuspended in 100 μL of wash buffer, and the fluorescence signal of the PE was read in the xMap® System using a Luminex® 100 IS cytometer (Bio-Rad). Levels of cytokines present in the samples were measured using cytokine standards provided with the bead kits (Invitrogen).

RNA extraction, reverse transcription, and real-time PCR- Total RNA were extracted from infected cell lysates using TRIZOL reagent (Invitrogen). 0.5 to 1 μg of RNA from each sample was treated with DNase I (Invitrogen), and subject to reverse transcription with oligo-dT primers (Invitrogen) using Superscript III reverse transcriptase (Invitrogen). The resulting cDNA was diluted 1 in 5, from which 2 μL of cDNA was used in a 25 μL real-time PCR reaction using cytokine gene-specific primers with the Jumpstart SYBR Green kit (Sigma) on an ABI 7900HT PRISM sequence detection system (Applied Biosystems). Primer sequences of specific cytokine genes are available upon request.

Interferon bioassay- Assays were performed as described previously with some modifications (Green *et al.*, 1980). Briefly, A549 cells were plated in 96-well microtiter plates (Corning) at a concentration 3.5 X 10^4 cell/well. Cells were treated for 6 hours with either positive control interferon beta (IFN-β) (Invitrogen), or 100 μL of gamma-irradiated supernatants from cells infected with NiV. Supernatants were removed, and each well was infected with ~200 PFU of Encephalomyocarditis virus (EMCV) for 16-20 hours in DMEM supplemented with 10% FBS. The supernatants were then removed, and the cells were stained with crystal violet solution for 5 minutes, washed once with PBS, and were scored for cytopathic effect (CPE) using a TECAN Sunrise plate reader at an absorbance of 600 nm. Uninfected cells stained with crystal violet served as a negative control for levels of CPE.

Interferon-β ELISA- Assays were performed according to manufacturer's protocols using a one-step sandwich human IFN-β ELISA kit (Invitrogen). Briefly, 100 μL of gamma irradiated supernatants from NiV infected cells were added to each well of a 96-well microplate pre-coated with affinity-purified polyclonal antibody to human IFN-β, along with 50 μL of horseradish peroxidase labeled-antibody for 2 hours on a plate shaker. After 3 wash steps, 100 μL of color developing solution was added to each well and incubated at room temperature for 30 minutes, upon which 100 μL of reaction stopper solution was added to each well. The absorbance of the reaction mixture in each well was read at 450 nm, with the reference wavelength set to 630 nm.

Chemotaxis Assay- Assays were performed according to manufacturer's instructions using the Chemicon QCM™ 5 μm 96-well cell migration kit (Millipore). In brief, THP-1 or JE-6.1 cells were centrifuged (1500 RPM, 5 minutes), re-suspended to a cell count of ~ 1.5 X 10^6/mL, and starved in DMEM without serum or chemoattractants for ~ 2 hours before being resuspended in fresh media and dispensed into the migration chamber at 1.2-1.5 X 10^5/well. Wells of a 96-well feeder tray were filled with 150 μL of either fresh endothelial growth media, or gamma-irradiated supernatants from NiV infected cells. After a 2 hour incubation of the chambers in the feeder wells, transmigrated cells in the feeder wells were transferred to a 96-well plate, while transmigrated cells attached to the outer surface of the incubation chamber were washed into a new feeder plate. These detached cells were then combined with the transmigrated cells in the 96-well plate, which were lysed with a lysis buffer mixed with CyQuant GR Dye, which is a DNA-binding fluorescent dye. The cell lysates were then transferred to white microtiter plates

(Thermo) and read with a Fluoroskan Ascent fluorescence plate reader using the 480/520 nm filter set (Thermo) with an integration time of 120msec. Fluorescence levels of the DNA-binding dye present in the cell lysates were indicative of the relative number of cells that migrated through the chamber. Supernatants taken from poly I:C stimulated endothelial cells served as positive controls for maximum induction of trans-membrane migration of THP-1 cells.

References

Aljofan, M., Porotto, M., Moscona, A. & Mungall, B. A. (2008). Development and validation of a chemiluminescent immunodetection assay amenable to high throughput screening of antiviral drugs for Nipah and Hendra virus. *J Virol Methods* **149**, 12-19.

Antony, V. B., Godbey, S. W., Kunkel, S. L., Hott, J. W., Hartman, D. L., Burdick, M. D. & Strieter, R. M. (1993). Recruitment of inflammatory cells to the pleural space. Chemotactic cytokines, IL-8, and monocyte chemotactic peptide-1 in human pleural fluids. *J Immunol* **151**, 7216-7223.

Ballew, H. C. (1986). Neutralization. In *Clinical Virology Manual*, pp. 187–199 Edited by S. S. a. G. J. Lancz. New York: Elsevier.

Cheng, G., Nazar, A. S., Shin, H. S., Vanguri, P. & Shin, M. L. (1998). IP-10 gene transcription by virus in astrocytes requires cooperation of ISRE with adjacent kappaB site but not IRF-1 or viral transcription. *J Interferon Cytokine Res* **18**, 987-997.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R. & Goodbourn, S. (2007). mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* **359**, 190-200.

Childs, K. S., Andrejeva, J., Randall, R. E. & Goodbourn, S. (2009). Mechanism of mda-5 Inhibition by paramyxovirus V proteins. *J Virol* **83**, 1465-1473.

Chua, K. B., Bellini, W. J., Rota, P. A., Harcourt, B. H., Tamin, A., Lam, S. K., Ksiazek, T. G., Rollin, P. E., Zaki, S. R., Shieh, W., Goldsmith, C. S., Gubler, D. J., Roehrig, J. T., Eaton, B., Gould, A. R., Olson, J., Field, H., Daniels, P., Ling, A. E., Peters, C. J., Anderson, L. J. & Mahy, B. W. (2000). Nipah virus: a recently emergent deadly paramyxovirus. *Science* **288**, 1432-1435.

Ciancanelli, M. J., Volchkova, V. A., Shaw, M. L., Volchkov, V. E. & Basler, C. F. (2009). Nipah virus sequesters inactive STAT1 in the nucleus via a P gene-encoded mechanism. *J Virol* **83**, 7828-7841.

Curran, J. & Kolakofsky, D. (1990). Sendai virus P gene produces multiple proteins from overlapping open reading frames. *Enzyme* **44**, 244-249.

Fisher, S. N., Vanguri, P., Shin, H. S. & Shin, M. L. (1995). Regulatory mechanisms of MuRantes and CRG-2 chemokine gene induction in central nervous system glial cells by virus. *Brain Behav Immun* **9**, 331-344.

Giorgi, C., Blumberg, B. M. & Kolakofsky, D. (1983). Sendai virus contains overlapping genes expressed from a single mRNA. *Cell* **35**, 829-836.

Glabinski, A. R. & Ransohoff, R. M. (1999). Chemokines and chemokine receptors in CNS pathology. *J Neurovirol* **5**, 3-12.

Goh, K. J., Tan, C. T., Chew, N. K., Tan, P. S., Kamarulzaman, A., Sarji, S. A., Wong, K. T., Abdullah, B. J., Chua, K. B. & Lam, S. K. (2000). Clinical features of Nipah virus encephalitis among pig farmers in Malaysia. *N Engl J Med* **342**, 1229-1235.

Gotoh, B., Komatsu, T., Takeuchi, K. & Yokoo, J. (2002). Paramyxovirus strategies for evading the interferon response. *Rev Med Virol* **12**, 337-357.

Green, J. A., Yeh, T. J. & Overall, J. C., Jr. (1980). Rapid, quantitative, semiautomated assay for virus-induced and immune human interferons. *J Clin Microbiol* **12**, 433-438.

Griffin, D. E. (1997). Cytokines in the brain during viral infection: clues to HIV-associated dementia. *J Clin Invest* **100**, 2948-2951.

Horvath, C. M. (2004). Silencing STATs: lessons from paramyxovirus interferon evasion. *Cytokine Growth Factor Rev* **15**, 117-127.

Hossain, M. J., Gurley, E. S., Montgomery, J. M., Bell, M., Carroll, D. S., Hsu, V. P., Formenty, P., Croisier, A., Bertherat, E., Faiz, M. A., Azad, A. K., Islam, R., Molla, M. A., Ksiazek, T. G., Rota, P. A., Comer, J. A., Rollin, P. E., Luby, S. P. & Breiman, R. F. (2008). Clinical presentation of nipah virus infection in Bangladesh. *Clin Infect Dis* **46**, 977-984.

Khuth, S. T., Akaoka, H., Pagenstecher, A., Verlaeten, O., Belin, M. F., Giraudon, P. & Bernard, A. (2001). Morbillivirus infection of the mouse central nervous system induces region-specific upregulation of MMPs and TIMPs correlated to inflammatory cytokine expression. *J Virol* **75**, 8268-8282.

Klein, R. S. (2004). Regulation of neuroinflammation: the role of CXCL10 in lymphocyte infiltration during autoimmune encephalomyelitis. *J Cell Biochem* **92**, 213-222.

Klein, R. S., Lin, E., Zhang, B., Luster, A. D., Tollett, J., Samuel, M. A., Engle, M. & Diamond, M. S. (2005). Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *J Virol* **79**, 11457-11466.

Kolakofsky, D., Roux, L., Garcin, D. & Ruigrok, R. W. (2005). Paramyxovirus mRNA editing, the "rule of six" and error catastrophe: a hypothesis. *J Gen Virol* **86**, 1869-1877.

Kunkel, E. J., Boisvert, J., Murphy, K., Vierra, M. A., Genovese, M. C., Wardlaw, A. J., Greenberg, H. B., Hodge, M. R., Wu, L., Butcher, E. C. & Campbell, J. J. (2002). Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am J Pathol* **160**, 347-355.

Kutsch, O., Oh, J., Nath, A. & Benveniste, E. N. (2000). Induction of the chemokines interleukin-8 and IP-10 by human immunodeficiency virus type 1 tat in astrocytes. *J Virol* **74**, 9214-9221.

Lane, T. E., Hardison, J. L. & Walsh, K. B. (2006). Functional diversity of chemokines and chemokine receptors in response to viral infection of the central nervous system. *Curr Top Microbiol Immunol* **303**, 1-27.

Lee, K. E., Umapathi, T., Tan, C. B., Tjia, H. T., Chua, T. S., Oh, H. M., Fock, K. M., Kurup, A., Das, A., Tan, A. K. & Lee, W. L. (1999). The neurological manifestations of Nipah virus encephalitis, a novel paramyxovirus. *Ann Neurol* **46**, 428-432.

Liston, P. & Briedis, D. J. (1995). Ribosomal frameshifting during translation of measles virus P protein mRNA is capable of directing synthesis of a unique protein. *J Virol* **69**, 6742-6750.

Lo, M. K., Harcourt, B. H., Mungall, B. A., Tamin, A., Peeples, M. E., Bellini, W. J. & Rota, P. A. (2009). Determination of the henipavirus phosphoprotein gene mRNA editing frequencies and detection of the C, V and W proteins of Nipah virus in virus-infected cells. *J Gen Virol* **90**, 398-404.

Lo, M. K. & Rota, P. A. (2008). The emergence of Nipah virus, a highly pathogenic paramyxovirus. *J Clin Virol* **43**, 396-400.

Loetscher, P., Seitz, M., Clark-Lewis, I., Baggiolini, M. & Moser, B. (1994). Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. *FASEB J* **8**, 1055-1060.

Mahalingam, S., Farber, J. M. & Karupiah, G. (1999). The interferon-inducible chemokines MuMig and Crg-2 exhibit antiviral activity In vivo. *J Virol* **73**, 1479-1491.

Manuse, M. J. & Parks, G. D. (2009). Role for the paramyxovirus genomic promoter in limiting host cell antiviral responses and cell killing. *J Virol* **83**, 9057-9067.

Melchjorsen, J., Jensen, S. B., Malmgaard, L., Rasmussen, S. B., Weber, F., Bowie, A. G., Matikainen, S. & Paludan, S. R. (2005). Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J Virol* **79**, 12944-12951.

Mikkelsen, S. S., Jensen, S. B., Chiliveru, S., Melchjorsen, J., Julkunen, I., Gaestel, M., Arthur, J. S., Flavell, R. A., Ghosh, S. & Paludan, S. R. (2009). RIG-I-mediated activation of p38 MAPK is essential for viral induction of interferon and activation of dendritic cells: dependence on TRAF2 and TAK1. *J Biol Chem* **284**, 10774-10782.

Nazar, A. S., Cheng, G., Shin, H. S., Brothers, P. N., Dhib-Jalbut, S., Shin, M. L. & Vanguri, P. (1997). Induction of IP-10 chemokine promoter by measles virus: comparison with interferon-gamma shows the use of the same response element but with differential DNA-protein binding profiles. *J Neuroimmunol* **77**, 116-127.

Owens, T., Babcock, A. A., Millward, J. M. & Toft-Hansen, H. (2005). Cytokine and chemokine inter-regulation in the inflamed or injured CNS. *Brain Res Brain Res Rev* **48**, 178-184.

Pace, E., Gjomarkaj, M., Melis, M., Profita, M., Spatafora, M., Vignola, A. M., Bonsignore, G. & Mody, C. H. (1999). Interleukin-8 induces lymphocyte chemotaxis into the pleural space. Role of pleural macrophages. *Am J Respir Crit Care Med* **159**, 1592-1599.

Parisien, J. P., Bamming, D., Komuro, A., Ramachandran, A., Rodriguez, J. J., Barber, G., Wojahn, R. D. & Horvath, C. M. (2009). A shared interface mediates paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2. *J Virol* **83**, 7252-7260.

Park, M. S., Shaw, M. L., Munoz-Jordan, J., Cros, J. F., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A. & Basler, C. F. (2003). Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol* **77**, 1501-1511.

Paton, N. I., Leo, Y. S., Zaki, S. R., Auchus, A. P., Lee, K. E., Ling, A. E., Chew, S. K., Ang, B., Rollin, P. E., Umapathi, T., Sng, I., Lee, C. C., Lim, E. & Ksiazek, T. G. (1999). Outbreak of Nipah-virus infection among abattoir workers in Singapore. *Lancet* **354**, 1253-1256.

Penido, C., Vieira-de-Abreu, A., Bozza, M. T., Castro-Faria-Neto, H. C. & Bozza, P. T. (2003). Role of monocyte chemotactic protein-1/CC chemokine ligand 2 on gamma delta T lymphocyte trafficking during inflammation induced by

lipopolysaccharide or Mycobacterium bovis bacille Calmette-Guerin. *J Immunol* **171**, 6788-6794.

Plumet, S., Herschke, F., Bourhis, J. M., Valentin, H., Longhi, S. & Gerlier, D. (2007). Cytosolic 5'-triphosphate ended viral leader transcript of measles virus as activator of the RIG I-mediated interferon response. *PLoS One* **2**, e279.

Rodriguez, J. J., Cruz, C. D. & Horvath, C. M. (2004). Identification of the nuclear export signal and STAT-binding domains of the Nipah virus V protein reveals mechanisms underlying interferon evasion. *J Virol* **78**, 5358-5367.

Rodriguez, J. J., Parisien, J. P. & Horvath, C. M. (2002). Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. *J Virol* **76**, 11476-11483.

Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P. & Basler, C. F. (2005). Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. *J Virol* **79**, 6078-6088.

Shaw, M. L., Garcia-Sastre, A., Palese, P. & Basler, C. F. (2004). Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. *J Virol* **78**, 5633-5641.

Shingai, M., Ebihara, T., Begum, N. A., Kato, A., Honma, T., Matsumoto, K., Saito, H., Ogura, H., Matsumoto, M. & Seya, T. (2007). Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J Immunol* **179**, 6123-6133.

Singh, A., Kulshreshtha, R. & Mathur, A. (2000). Secretion of the chemokine interleukin-8 during Japanese encephalitis virus infection. *J Med Microbiol* **49**, 607-612.

Stamatovic, S. M., Shakui, P., Keep, R. F., Moore, B. B., Kunkel, S. L., Van Rooijen, N. & Andjelkovic, A. V. (2005). Monocyte chemoattractant protein-1 regulation of blood-brain barrier permeability. *J Cereb Blood Flow Metab* **25**, 593-606.

Vanguri, P. & Farber, J. M. (1994). IFN and virus-inducible expression of an immediate early gene, crg-2/IP-10, and a delayed gene, I-A alpha in astrocytes and microglia. *J Immunol* **152**, 1411-1418.

Wong, K. T., Shieh, W. J., Kumar, S., Norain, K., Abdullah, W., Guarner, J., Goldsmith, C. S., Chua, K. B., Lam, S. K., Tan, C. T., Goh, K. J., Chong, H. T., Jusoh, R., Rollin, P. E., Ksiazek, T. G. & Zaki, S. R. (2002). Nipah virus infection: pathology and pathogenesis of an emerging paramyxoviral zoonosis. *Am J Pathol* **161**, 2153-2167.

Zhang, B., Chan, Y. K., Lu, B., Diamond, M. S. & Klein, R. S. (2008). CXCR3 mediates region-specific antiviral T cell trafficking within the central nervous system during West Nile virus encephalitis. *J Immunol* **180**, 2641-2649.

Zhang, Y., Luxon, B. A., Casola, A., Garofalo, R. P., Jamaluddin, M. & Brasier, A. R. (2001). Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. *J Virol* **75**, 9044-9058.

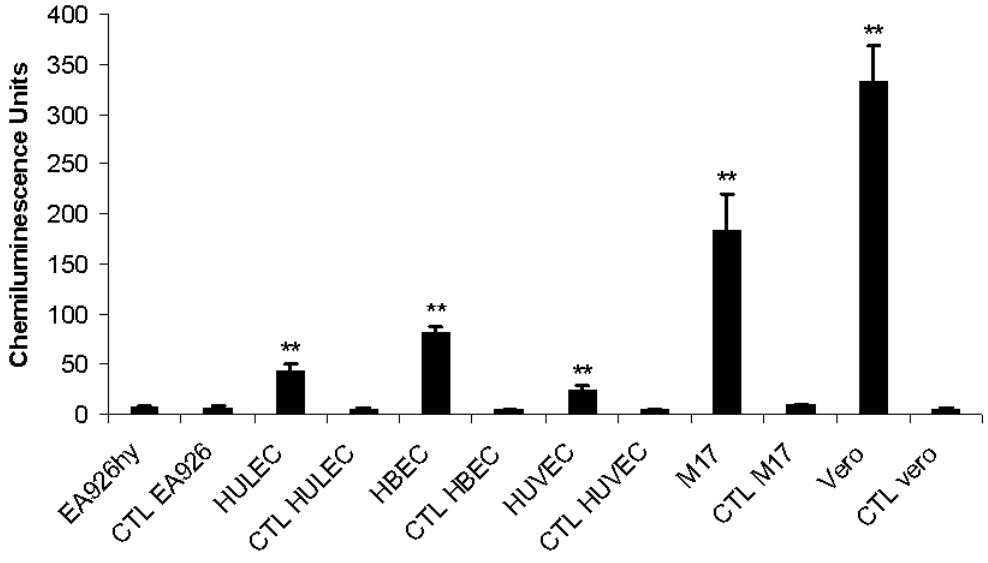
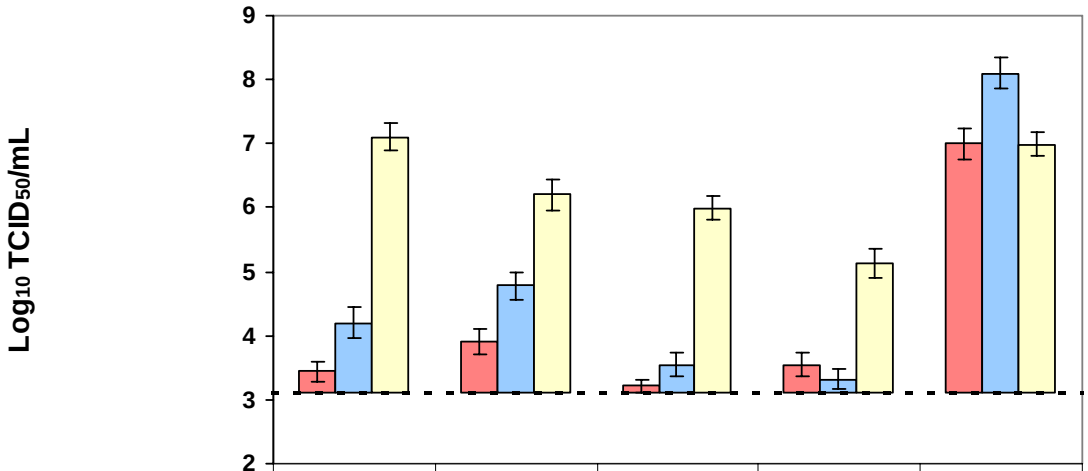


Figure 1. **Levels of NiV antigen increases at a greater rate in neuronal cells compared with endothelial cells.** In this immunolabeling assay, endothelial and neuronal cells were infected for 16 hours, then fixed, washed, and incubated with rabbit anti-NiV N antibodies. A mouse anti-rabbit IgG antibody conjugated with horseradish peroxidase was used as a means to measure relative levels of NiV antigen. CTL indicates uninfected controls for each respective cell line. N = 5. ** ($P < 0.001$) (Relative to levels of chemiluminescence detected from respective mock infected endothelial/neuronal cell types)

A.



	HUVEC	HULEC	LYMEC	HBEC	M17
16h	3.44	3.91	3.21	3.55	7
24h	4.2	4.78	3.55	3.32	8.1
48h (32h M17)	7.1	6.2	5.99	5.14	6.99

B.

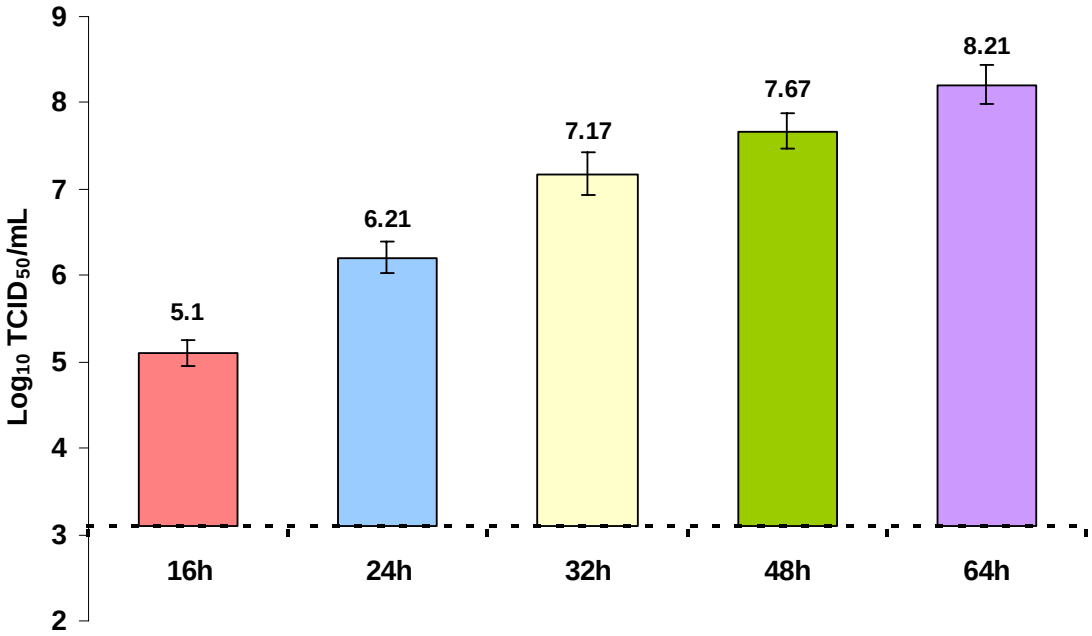


Figure 2. **Single-step growth curve of NiV infection of endothelial and neuronal cells**. A) Supernatants taken from NiV infected endothelial cells and neuronal cells (infected at MOI=1) were serially diluted before being used to infect Vero cells to determine the $TCID_{50}$/mL at the indicated time-points. B) Single-step growth curve of NiV infection of HUVECs (MOI=2). The dotted line delineates the limit of detection (3.1). N = 9 for both growth curve experiments.

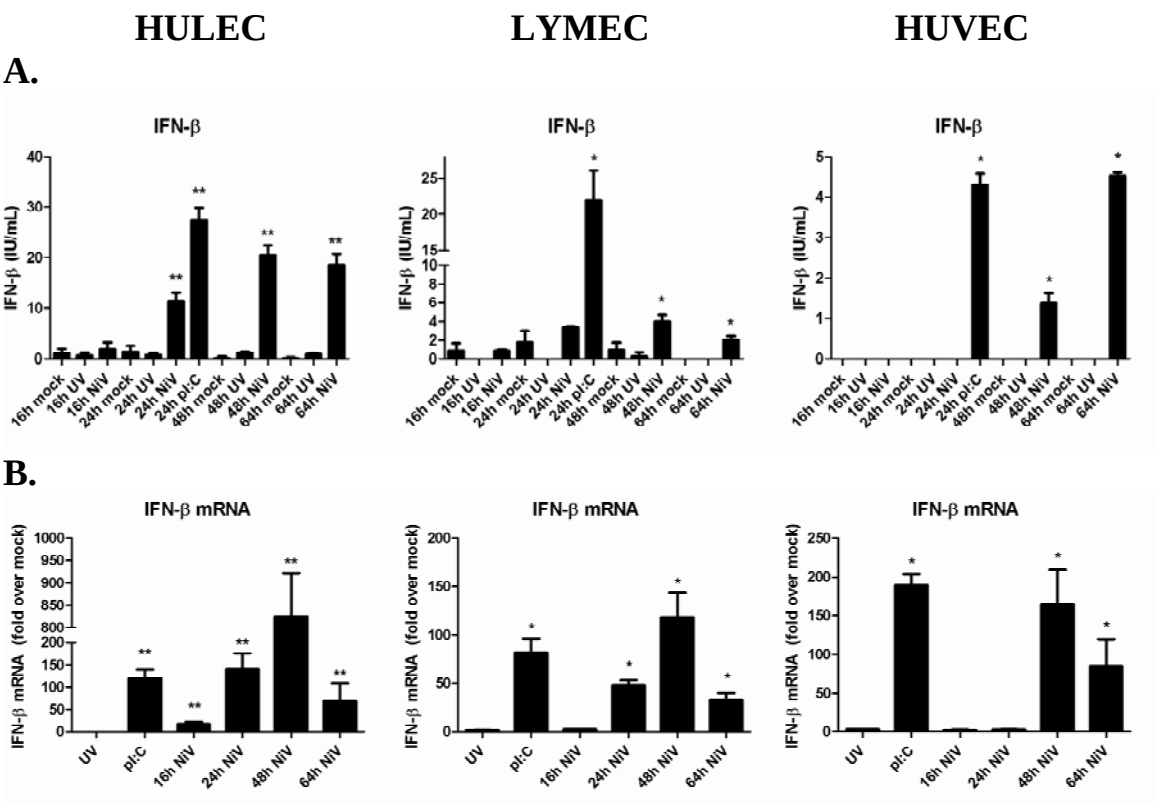


Figure 3. **NiV infection of endothelial cells induces IFN-β production.** A) IFN-β ELISA were performed on of supernatants taken from NiV infected lung, lymphatic, and umbilical vein endothelial cells at indicated time-points. B) Levels of IFN-β mRNA transcription in NiV infected lung, lymphatic, and umbilical vein endothelial cells were measured by Real-time PCR, which was performed on cDNA generated from extracted total cellular RNA using oligo-$dT_{12\text{-}18}$ primers. N = 3. Labels above the graphs correspond to the data obtained from each endothelial cell type. *($P < 0.05$); **($P < 0.01$) (A, C, E: Relative to levels of IFN-β detected in supernatants from mock infected cells at corresponding time point; B, F, F: Relative to mRNA transcript levels in mock infected cells at corresponding time point)

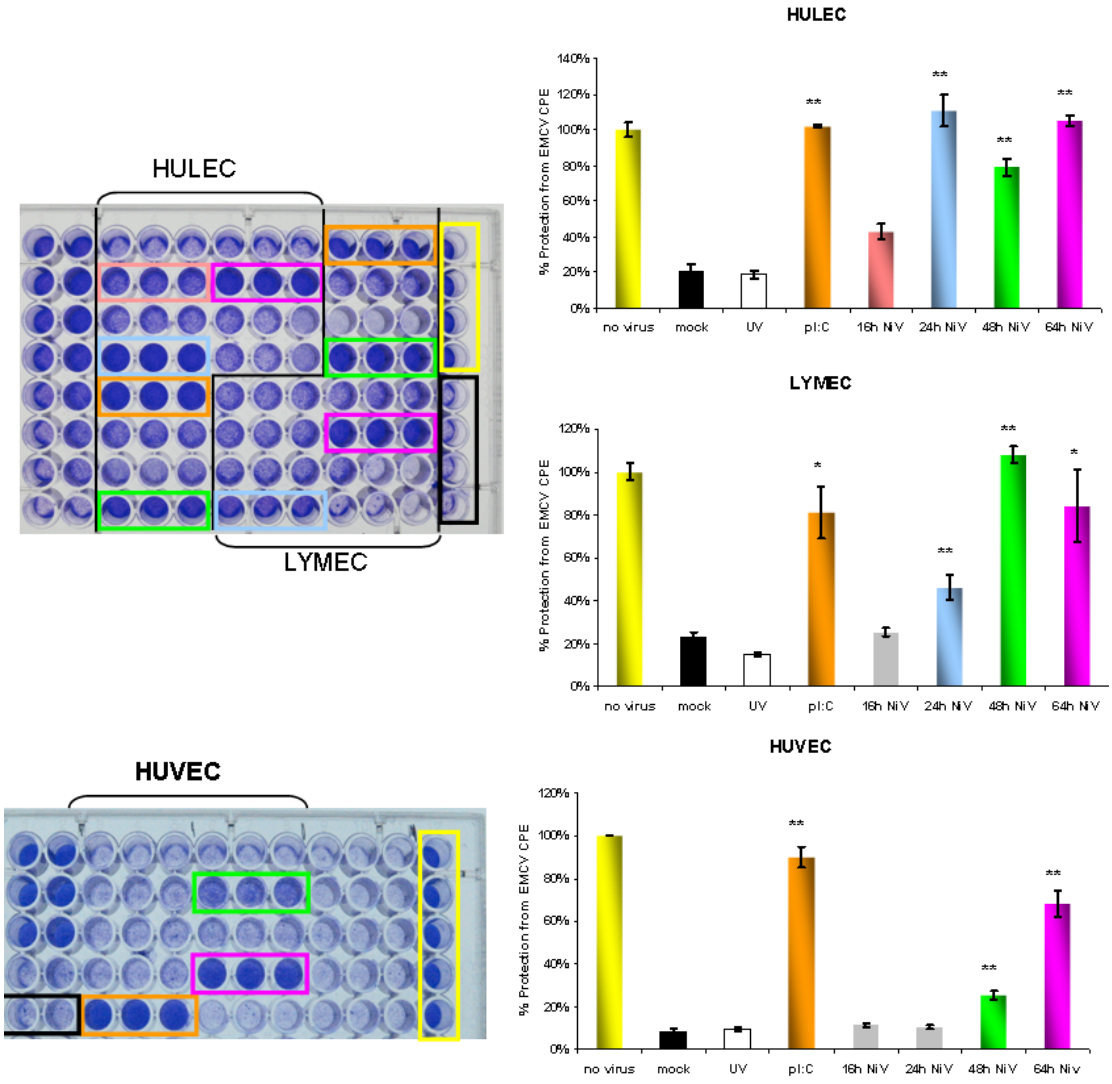


Figure 4. **Supernatants from NiV infected endothelial cells contain functional IFN-β as assayed in an Encephalomyocarditis virus (EMCV) Cytopathic Effect (CPE) assay**. A549 human respiratory epithelial cells were pre-treated with gamma-irradiated supernatants from NiV infected endothelial cells for 6 hours, and then infected with ~ 200 PFU/well for 16-20 hours before being stained with crystal violet solution. Stained 96-well plates of CPE assays performed using supernatants from endothelial cell supernatants indicated above/below each portion of the plate. Colored boxes surrounding wells correspond to colored bars in the graphs for assays performed with supernatants from each respective NiV infected endothelial cell type. N = 3. Relative to cells treated with supernatants from mock infected cells at corresponding time points **($P < 0.01$); *($P < 0.05$)

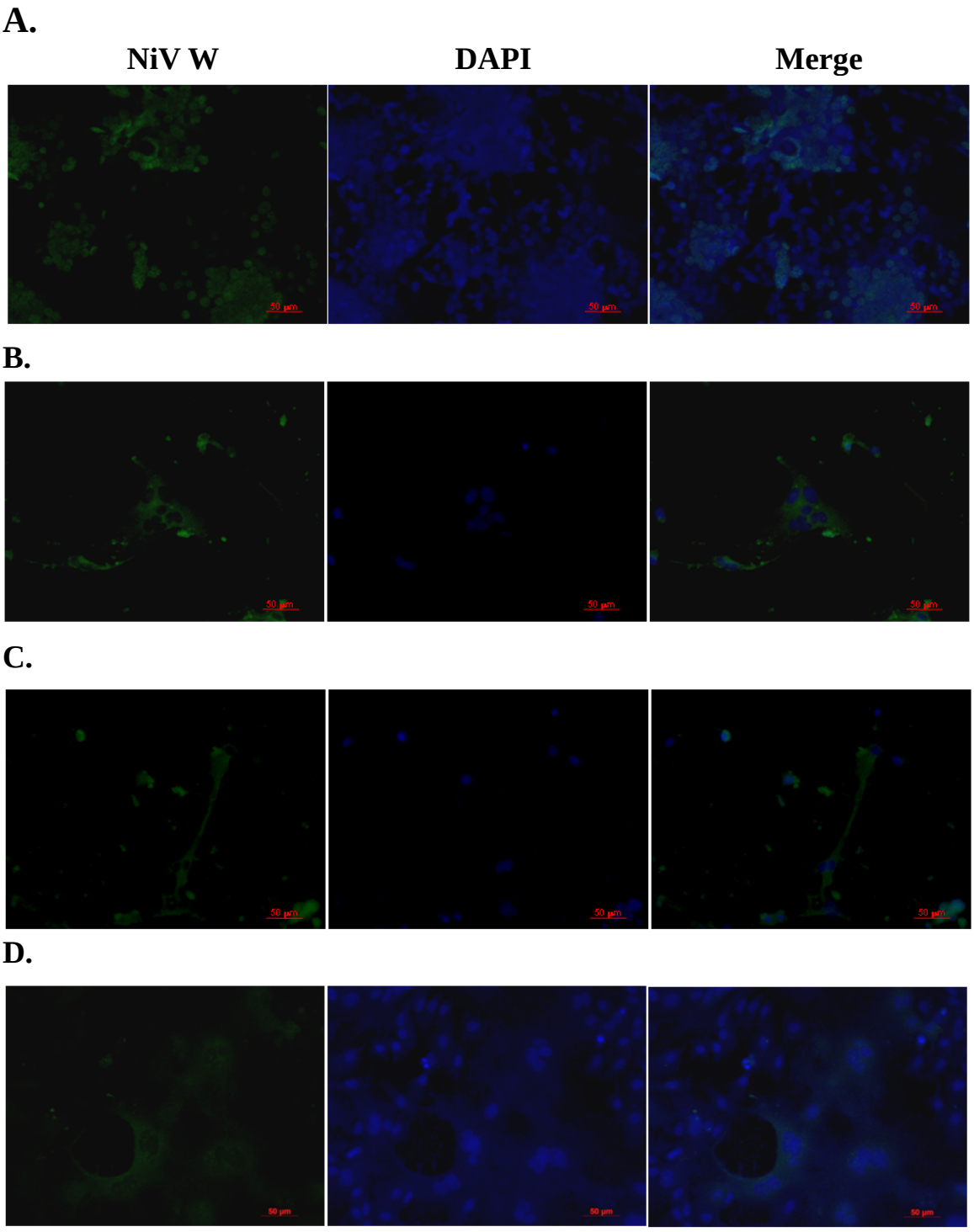
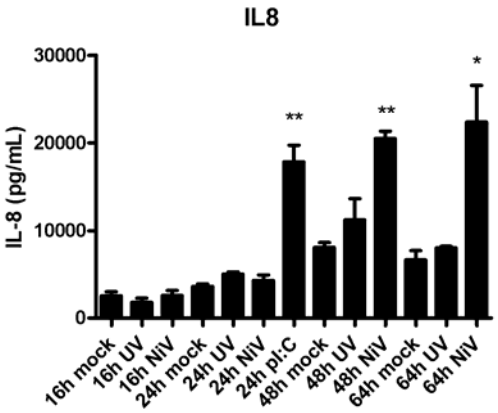


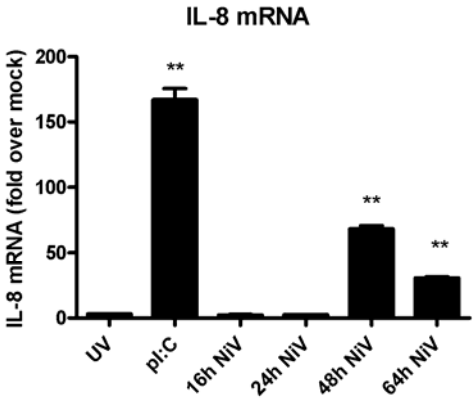
Figure 5. **Differential subcellular localization of NiV W protein in NiV infected neuronal and endothelial cells.** NiV W was detected in infected A) M17 neuronal cells, B) HULEC, C) LYMEC, and D) HUVEC. Magnification: 20X

Supernatant **mRNA**

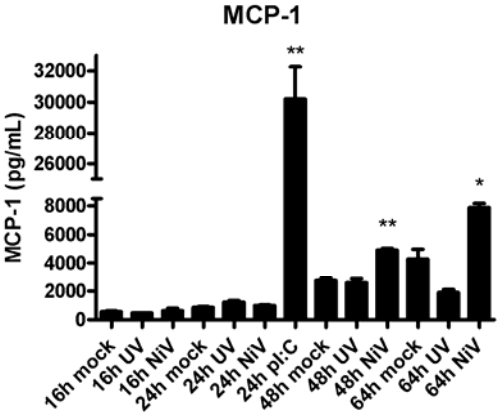
A.



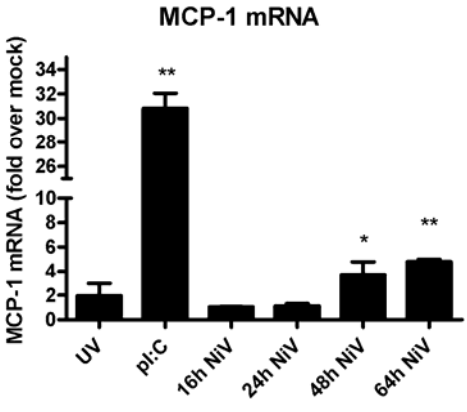
B.



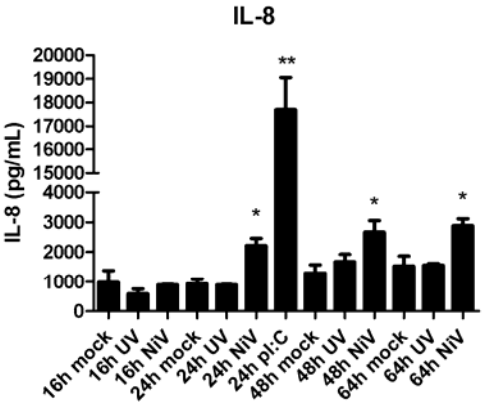
C.



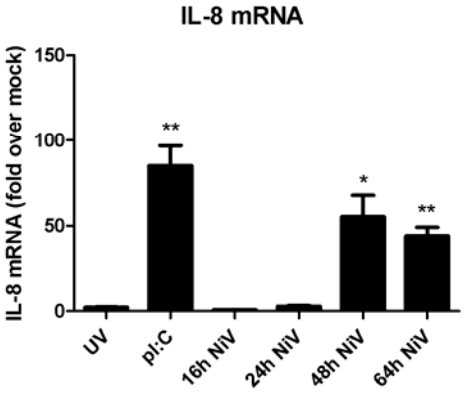
D.



E.



F.



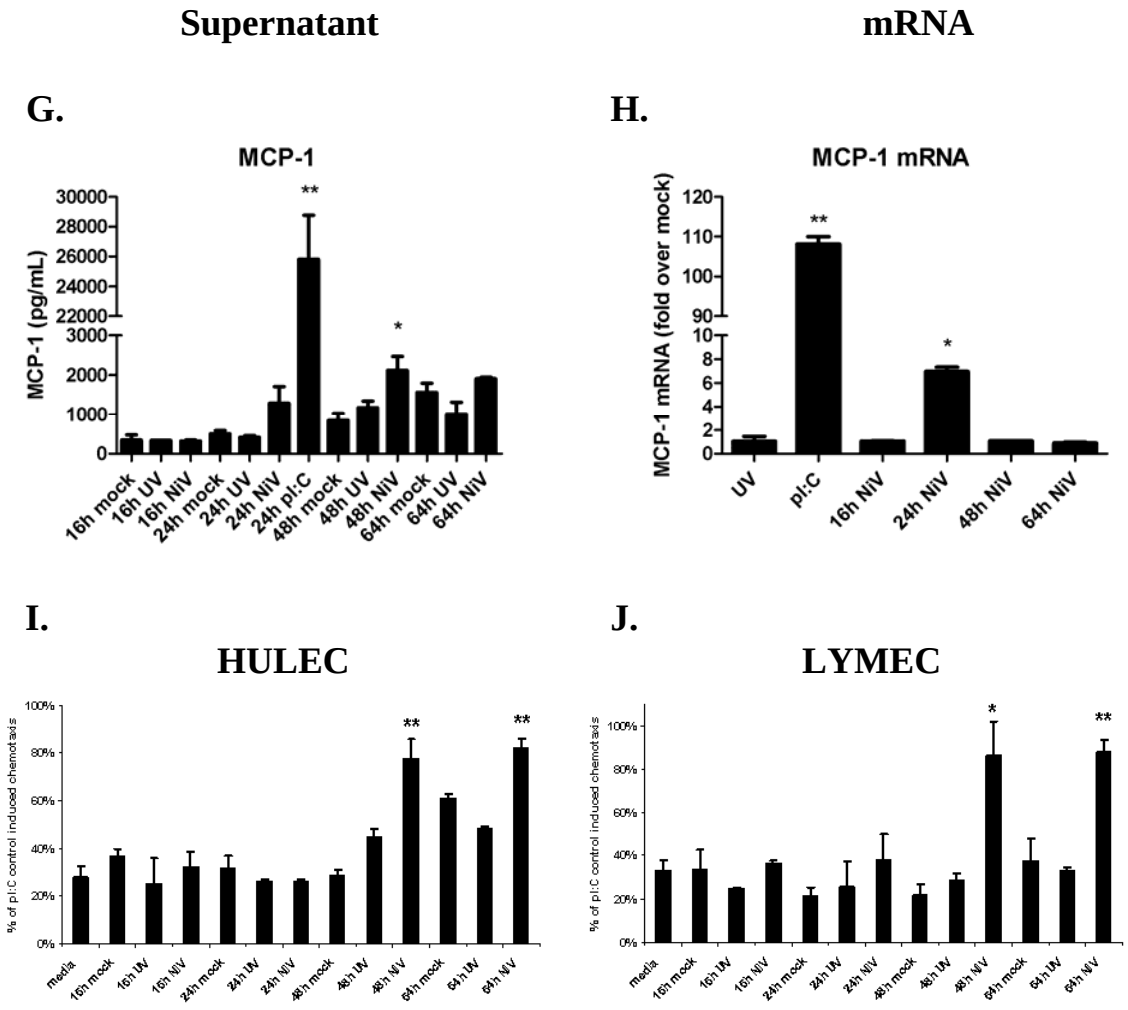


Figure 6. **NiV infection induces endothelial cell production of inflammatory chemokines associated with increased monocyte chemotaxis**. Luminex bead assays were performed on gamma-irradiated NiV infected HULEC (A, C) and LYMEC (E, G) supernatants to detect the presence of IL-8 and MCP-1. Levels of IL-8 and MCP-1 mRNA transcription in NiV infected HULEC (B, D) and LYMEC (F, H) were measured by Real-time PCR, which was performed using cDNA generated from extracted total cellular RNA using oligo-dT_{12-18} primers. THP-1 monocyte chemotaxis assay (I & J). Levels of THP-1 monocytic cell chemotaxis across a 5 μm pore membrate after 2 hours of exposure to above mentioned supernatants relative to supernatants from poly-I:C stimulated endothelial cells. N = 3. **($P < 0.01$), *($P < 0.05$). (A, C, E, G: Relative to chemokine levels in mock treated endothelial cell supernatants at corresponding time point; B, D, F, H: Relative to levels of mRNA from mock infected endothelial cells at corresponding time point; I-J: Relative to levels of chemotaxis induced by mock treated endothelial cell supernatants at corresponding time point)

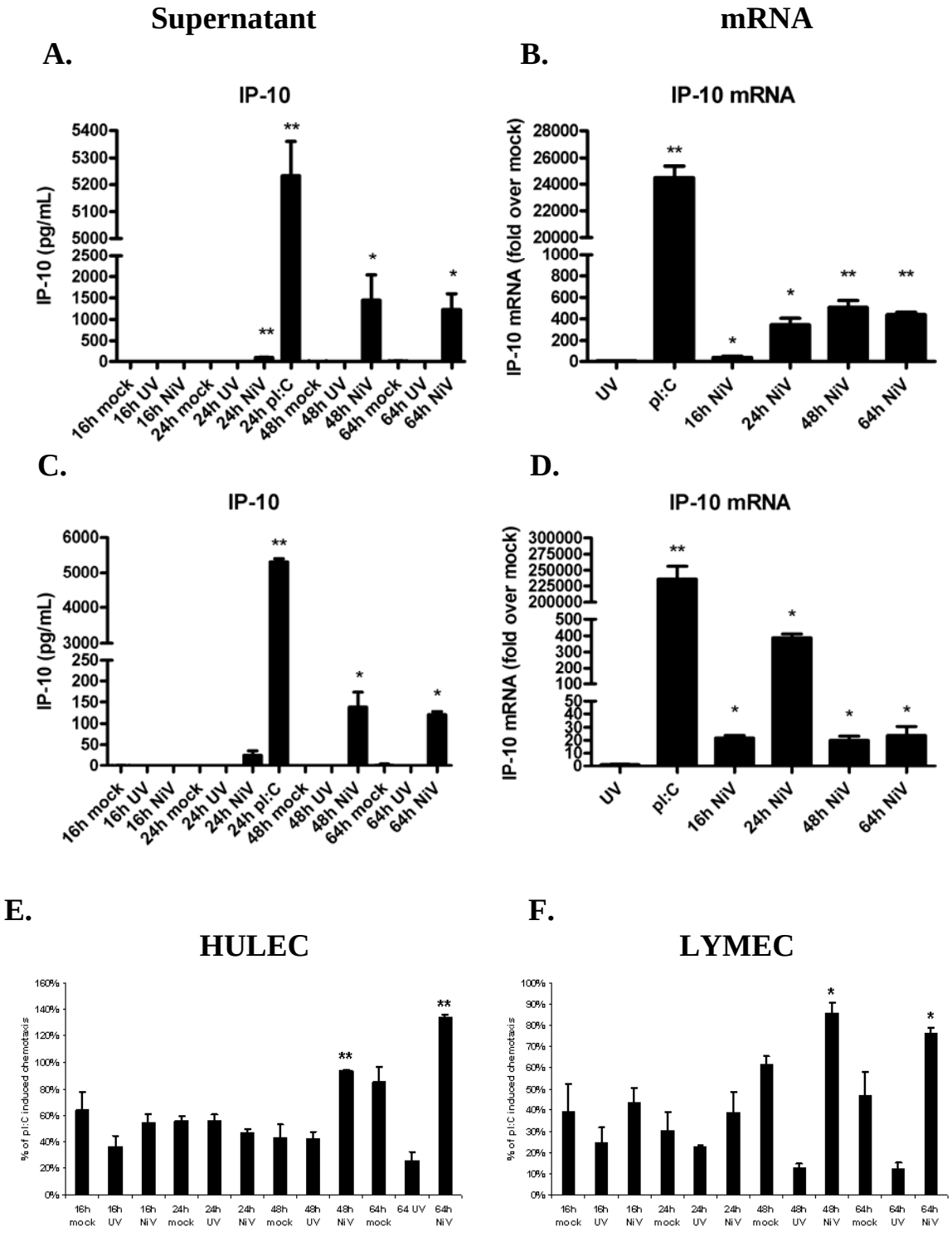


Figure 7. **NiV infection induces endothelial cell production of inflammatory cytokines associated with increased T lymphocyte chemotaxis.** Luminex bead assays were performed on gamma-irradiated NiV infected A) HULEC and C) LYMEC supernatants to detect the presence of IP-10 (CXCL10). Levels of IP-10 mRNA

transcription in NiV infected (B) HULEC and (D) LYMEC were measured by Real-time PCR, which was performed using cDNA generated from extracted total cellular RNA using oligo-dT_{12-18} primers. Levels of JE-6.1 T-lymphocyte chemotaxis across a 5 μm pore membrane after 2 hours of exposure to NiV infected (E) HULEC or (F) LYMEC supernatants relative to supernatants from poly-I:C stimulated endothelial cells. N = 3. **($P < 0.01$), *($P < 0.05$). (A, C: Relative to mock infected endothelial cell supernatants at corresponding time point) (B, D: Relative to mock infected endothelial cell mRNA transcript levels at corresponding time point)

Chapter 4

Discussion and Conclusions

Since the initial outbreak of Nipah (NiV) in Malaysia just over a decade ago, a significant body of work has been performed in characterizing the actions of individual NiV genes in the context of eukaryotic plasmid expression studies. Due to the lack of approved therapeutic antivirals against this highly virulent pathogen, biosafety level 4 containment facilities are required in order to work with live NiV, which has partially hindered the ability of researchers to address questions regarding NiV replication and pathogenesis in physiologically relevant contexts. With the increasing number of BSL-4 facilities being built around the world, and the availability of NiV reverse genetic systems, we expect that the body of knowledge in regards to NiV replication and pathogenesis will significantly increase in the near future (Ciancanelli *et al.*, 2009; Yoneda *et al.*, 2006).

In this report, we determined the phosphoprotein (P) gene mRNA editing frequencies of Nipah Malaysia, Nipah Bangladesh, and Hendra viruses. We demonstrated that these three viruses edit approximately two-thirds of their P gene mRNAs, resulting in an overall relative abundance of P gene mRNA ratio of 2:1:1 encoding the NiV P, V, and W proteins respectively. Subsequent to our publication of this study, another group confirmed the high expression levels of NiV V and W in NiV infected cells, and that the expression of these proteins began very early in infection (Kulkarni *et al.*, 2009; Lo *et al.*, 2009). Henipaviruses edit their P genes at higher frequencies than most other viruses in the family *Paramyxoviridae*, with the exception of bovine parainfluenza virus 3 (BPIV3), which edits its P gene mRNA at similar frequencies to henipaviruses (Pelet *et al.*, 1991). Our study was the initial characterization of the subcellular localizations of the P gene products in the context of

live NiV infection. We found that contrary to the homogenous distribution of the NiV P protein shown in plasmid expression studies, the NiV P gene was punctuate in its distribution in the nucleus, and tended to concentrate towards the plasma membrane. The P proteins of paramyxoviruses serve as molecular chaperones between the Large (L) polymerase protein and the nucleoprotein (N)-encapsidated genome (Curran *et al.*, 1995; Horikami *et al.*, 1992; Horikami *et al.*, 1994). While the interaction domains between the N and P proteins have previously been mapped in the context of a protein blotting overlay assay, the domains required for replication in a NiV minigenome assay have only partially been mapped in the P gene (Chan *et al.*, 2004; Ciancanelli *et al.*, 2009). One future avenue in studying NiV replication could focus on characterizing and mapping the domains in the N gene required for RNA binding as opposed to the domains required for N-P or potential N-L interactions. The role of the P gene accessory proteins may potentially factor into this as well. Our group has previously shown that exogenous expression of NiV C, V, and W inhibits replication of a NiV minigenome (Sleeman *et al.*, 2008). Given the distinct subcellular localizations of each protein, it is plausible to suggest that the mechanisms underlying the inhibition caused by each protein are possibly different. Future studies utilizing reverse genetic systems can be manipulated to mutate the mRNA editing site in the P gene so as to abrogate the viral editing of P gene mRNA coding for the V and W proteins. If this P gene mRNA mutant editing site virus can be rescued from cDNA, its growth characteristics should be measured to determine the contribution (or detraction) of V and W to levels of viral replication.

Several significant studies have focused on the interaction of the NiV P gene products with elements of the host innate immune response. In the context of plasmid

transfections, the shared N-terminus between the NiV P, V, and W proteins have been shown to interact with signal transduction activator of transcription (STAT) 1, sequestering it in high molecular weight complexes, preventing its phosphorylation, and thereby subverting the type I interferon (IFN) signaling pathway (Rodriguez *et al.*, 2004; Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). A more recent study has shown that a mutant NiV carrying a mutated P gene that could not interact with STAT-1 could not inhibit STAT-1 phosphorylation upon treatment with exogenous IFN-β in Vero cells. This mutant virus however, was constructed in the context of a C protein knockout, and there was no detectable difference in growth kinetics between the C protein knockout virus and the STAT-1 binding mutant virus. It was the C protein knockout virus (as well as the STAT-1 binding mutant virus) that showed a substantial deficiency in viral replication compared to the wild type NiV rescued from cDNA both in IFN competent (293T) and IFN incompetent (Vero E6) cells (Ciancanelli *et al.*, 2009). That particular study demonstrated the importance of the NiV C protein in virus replication. However, the mechanism by which NiV C promoted viral replication is unclear. Plasmid expressed NiV C was shown to partially rescue Newcastle disease virus replication in chick embryo fibroblast cells, but did not block the type I IFN signaling pathway (Caignard *et al.*, 2009; Lo & Rota, 2008; Park *et al.*, 2003). The C proteins of viruses in the subfamily *Paramyxovirinae* have been shown to inhibit antiviral responses and to serve as virulence factors; the mechanisms by which they act however, are distinct (Escoffier *et al.*, 1999; Garcin *et al.*, 1997; Garcin *et al.*, 1999; Gotoh *et al.*, 2001). The Sendai virus (SeV) C proteins inhibit both type I IFN induction as well as IFN signaling, while the measles virus (MeV) C protein modulates virus replication to limit IFN induction due to virus

production of pathogen associated molecular patterns (PAMPs) (Nakatsu *et al.*, 2006; Nakatsu *et al.*, 2008). The Rinderpest virus C protein also inhibits IFN-β induction (Boxer *et al.*, 2009). The SeV C protein has been shown to have a function in facilitating virion release (Sugahara *et al.*, 2004). Given that the decrease in viral titer in a mutant NiV with a C protein knockout occurs in both IFN competent and incompetent cells, it is possible that perhaps the NiV C has a role besides circumventing the innate antiviral response (Ciancanelli *et al.*, 2009). Among the four NiV P gene derived proteins, the NiV C protein is the least-studied. Further insight into the action(s) of the NiV C protein can be obtained in several ways. One method is to determine whether it localizes to particular cellular compartments during infection. In our study, we were able to qualitatively describe the subcellular localization of NiV C in the context of infection, but did not proceed to determine whether it trafficked to a specific cell compartment/ or organelle. Determining the exact cellular compartments where NiV C localizes if possible, would likely contribute to identifying possible functions of this protein. Another method is to immunoprecipitate NiV C from infected cells, and to use mass spectrometry to determine the presence of any cellular factors that co-precipitate with NiV C. Our study not only demonstrated the expression of the P gene products in NiV infected cells, but detected the presence of NiV C, V, and W in sucrose gradient purified NiV. The presence of these proteins in the virion may give NiV an advantage in blocking activation of any pre-existing PRRs present in the host cell cytoplasm once NiV fuses its membrane upon entry and releases its contents into the cell. It has already been shown that a mutant NiV lacking C protein expression replicates to a significantly lower titer than a wild-type virus (Ciancanelli *et al.*, 2009). If it is possible to rescue and sucrose

gradient purify a mutant NiV that does not contain any C, V, or W proteins in its virions, it would be interesting to see whether there is a significantly delayed rate of replication compared to wild-type and mutant C viruses.

The nuclear localization of NiV W is critical for its enhanced ability to block IRF-3 activation. Although we have demonstrated the nuclear localization of NiV W in infected Vero cells, our study of NiV infection of primary endothelial cells demonstrated that the majority of detected NiV W was located in the cytoplasm as opposed to its nuclear localization in NiV infected neuronal cells. In accordance with this finding, we found that every endothelial cell type infected with NiV was able to produce a functional IFN-β response, while there was no detectable IFN-β generated in the neuronal cells. These results indicate an additional intracellular mechanism by which endothelial cells can slow viral replication by potential protein-protein interactions with specific viral proteins. We have demonstrated that in NiV infected Vero cells, NiV W protein coprecipitates with Karyopherin α3 (KPNA3), which is part of the nuclear import machinery that recognizes the nuclear localization signal in NiV W (Lo *et al.*, 2009; Shaw *et al.*, 2005). It will be interesting to determine whether this interaction is disrupted in NiV infected endothelial cells. If this interaction indeed is blocked, mass spectrometry experiments should be performed on immunoprecipitated lysates of NiV W and Karyopherin α3 from NiV infected endothelial cells to determine potential interacting cellular proteins that may block their interaction. Characterizing these putative cellular factors would not only shed light on the endothelial host cell response to NiV infection, but would perhaps lead to discoveries of novel cell-type specific antiviral mechanisms.

Our observation of the ability of primary endothelial cells to produce IFN-β in response to NiV infection leads us to question which pattern recognition receptors were responsible for this observed induction. One possibility is that the exclusion of NiV W from the cytoplasm potentially allowed for IRF-3 activation via the Toll-like-receptor (TLR) 3 pathway. Double-stranded RNA (dsRNA) is the typical activating ligand of TLR-3, but it has been questioned as to whether negative-sense RNA viruses generate dsRNA in the course of infection (Alexopoulou *et al.*, 2001; Weber *et al.*, 2006). Another possibility is that the RNA helicase RIG-I is responsible for detecting NiV replication and for generating the IFN-β response. RIG-I has been shown to generate an IFN response against the 5' tri-phosphorylated cytosolic leader of MeV RNA (Plumet *et al.*, 2007). Extensive studies performed on defining the structural requirements for RIG-I recognition of RNA demonstrated that RIG-I requires double-stranded structures in conjunction with 5' triphosphorylation. It was shown that the leader sequence of Rabies virus in its native panhandle form could induce a type I IFN response, while a mismatched form of the panhandle leader RNA transcript could not do so (Plumet *et al.*, 2007; Schlee *et al.*, 2009; Schmidt *et al.*, 2009). It has also been demonstrated that the wild type genomic promoter sequences of parainfluenza virus simian virus 5 (SV5) have evolved to minimize detection by RIG-I, while certain variant strains SV5 with sequence mutations in the genomic promoter would induce high levels of inflammatory and antiviral cytokines. Determining whether or not the genomic leader and promoter sequences of NiV are recognized by RIG-I will productively contribute to our understanding of the innate antiviral response observed in the endothelial cells. It is possible that different PRRs will be responsible for detecting varied PAMPs generated by

NiV replication, depending on the cell type. It has been shown that while non-immune cells depend mostly on RIG-I and mda-5 to detect viral infections, myeloid cells utilize TLRs 7 and 8 along with other dsRNA dependent mechanisms that are independent of RIG-I, TLR3, or protein kinase R (Melchjorsen *et al.*, 2005; Yoneyama *et al.*, 2005). Endothelial cells have been shown to express basal levels of several PRRs including TLRs, and it will be interesting to determine which of these PRRs are responsible for mounting an antiviral response against NiV (Breslin *et al.*, 2008; Park *et al.*, 2007; Pegu *et al.*, 2008) We have demonstrated that the human endothelial cells used in our study can generate significant amounts of antiviral and inflammatory cytokines in response to exogenously added poly I:C, which indicates the expression of TLR-3 in these cells. Using small interfering RNAs packaged in lentivirus particles to knock down the expression of various PRRs present in endothelial cells will be useful to distinguish the most important elements required for the observed immune response against NiV infection. Several studies have shown that paramyxovirus V proteins including that of NiV bind to the RNA helicase mda-5 and preclude its activation by dsRNA (Andrejeva *et al.*, 2004; Childs *et al.*, 2007; Childs *et al.*, 2009; Parisien *et al.*, 2009). It will be useful to determine whether cytoplasmic mda-5 co-localizes with NiV V protein during infection of endothelial cells.

In our study, we demonstrated that primary endothelial cells generated a significant inflammatory chemokine response to NiV infection, and that the inflammatory mediators secreted into the cellular supernatant was able to induce an increase in monocyte and T-lymphocyte chemotaxis. These findings provide a preliminary *in vitro* model to explain the extensive vasculitis seen in human infections. In order to pinpoint

which inflammatory chemokines are responsible for upregulating chemotaxis, future studies should utilize blocking antibodies against potential chemokines and their corresponding receptors. Since many chemokine receptors can typically bind to more than one chemokine, receptor-blocking antibodies will help narrow down the candidates before using blocking antibodies specific for each chemokine (Lane *et al.*, 2006).

As more knowledge is revealed in regards to the molecular mechanisms of pathogenesis in the primary target cells of NiV infection as observed in pathology reports, we are lead to ask questions of origins: Where is/are the initial primary sites of NiV replication in human infection, and how does NiV establish infection in those sites? Given the tropism of NiV across five mammalian terrestrial orders (Lo & Rota, 2008), several animal models of NiV infection have been developed, including golden hamsters, guinea pigs, ferrets, cats, and pigs (Georges-Courbot *et al.*, 2006; Mungall *et al.*, 2007; Pallister *et al.*, 2009; Torres-Velez *et al.*, 2008; Weingartl *et al.*, 2005). A recent study of NiV infected pigs indicated an increased susceptibility to bacterial infections. In conjunction with the observation of significant lymphoid depletion in the lymph nodes and *in vitro* NiV replication in porcine peripheral blood mononuclear cells (PBMCs), this study pointed to the possibility of NiV-induced immune suppression (Berhane *et al.*, 2008). In human cases of NiV infection, there was also extensive necrosis and lymphoid depletion in the vicinity of the periarteriolar sheath of the spleen and lymph nodes. The relative absence of viral antigen in autopsies in spite of the extensive necrosis observed possibly implicates lymphoid tissue as an early site of NiV replication (Wong *et al.*, 2002). We suggest that these macroscopic observations warrant future studies of the ability of NiV to infect immune cells in order to determine their capacity to serve as

productive initial reservoirs for virus replication. If NiV productively infects immune cells and can grow to relatively high titers, new questions arise depending on which immune cell types are infected. Does NiV infection affect levels of antigen presentation by professional antigen presenting cells (APCs)? Does NiV affect the ability of APCs to stimulate a robust T-lymphocyte response? Are there certain immune cell types that are crucial to generating a protective response to NiV? These questions can be addressed to a certain extent in the context of *in vitro* infections of human lymphocytes, PBMCs and the like, but eventually small animal models will be invaluable in modeling the progression of infection from the initial stages of establishment of infection to the end stages of pathology.

Our work has significantly contributed to advancing the knowledge of NiV replication and pathogenesis at the molecular level. This is the first report characterizing the P gene mRNA editing frequencies and the expression of the four P gene derived proteins of NiV. We also demonstrated for the first time the distinct subcellular localization of the NiV P, V, W, and C proteins, and showed that the V, W, and C proteins are all present in purified NiV virions. In the context of viral infection, we confirmed previous results from plasmid expression studies indicating an interaction between NiV W and KPNA3. This is also the first report to characterize the initial antiviral and inflammatory endothelial cell response to NiV infection. We demonstrated that contrary to NiV infections in Vero and M17 neuronal cells, the majority of NiV W protein localized to the cytoplasm, and showed that this correlated with the ability of endothelial cells to generate a functional IFN-β response. This is the first study in which inflammatory cytokine chemokine response was measured from physiologically relevant

primary endothelial cells and the first to demonstrate a significant induction of monocyte and T-lymphocyte chemotaxis by NiV infection. Future studies are required to identify the exact chemokines responsible for inducing the observed chemotaxis, and to define the molecular pathways by which these chemokines are induced.

References

Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-738.

Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S. & Randall, R. E. (2004). The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci U S A* **101**, 17264-17269.

Berhane, Y., Weingartl, H. M., Lopez, J., Neufeld, J., Czub, S., Embury-Hyatt, C., Goolia, M., Copps, J. & Czub, M. (2008). Bacterial infections in pigs experimentally infected with nipah virus. *Transbound Emerg Dis* **55**, 165-174.

Boxer, E. L., Nanda, S. K. & Baron, M. D. (2009). The rinderpest virus non-structural C protein blocks the induction of type 1 interferon. *Virology* **385**, 134-142.

Breslin, J. W., Wu, M. H., Guo, M., Reynoso, R. & Yuan, S. Y. (2008). Toll-like receptor 4 contributes to microvascular inflammation and barrier dysfunction in thermal injury. *Shock* **29**, 349-355.

Caignard, G., Komarova, A. V., Bourai, M., Mourez, T., Jacob, Y., Jones, L. M., Rozenberg, F., Vabret, A., Freymuth, F., Tangy, F. & Vidalain, P. O. (2009). Differential regulation of type I interferon and epidermal growth factor pathways by a human Respirovirus virulence factor. *PLoS Pathog* **5**, e1000587.

Chan, Y. P., Koh, C. L., Lam, S. K. & Wang, L. F. (2004). Mapping of domains responsible for nucleocapsid protein-phosphoprotein interaction of Henipaviruses. *J Gen Virol* **85**, 1675-1684.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R. & Goodbourn, S. (2007). mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. *Virology* **359**, 190-200.

Childs, K. S., Andrejeva, J., Randall, R. E. & Goodbourn, S. (2009). Mechanism of mda-5 Inhibition by paramyxovirus V proteins. *J Virol* **83**, 1465-1473.

Ciancanelli, M. J., Volchkova, V. A., Shaw, M. L., Volchkov, V. E. & Basler, C. F. (2009). Nipah virus sequesters inactive STAT1 in the nucleus via a P gene-encoded mechanism. *J Virol* **83**, 7828-7841.

Curran, J., Marq, J. B. & Kolakofsky, D. (1995). An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. *J Virol* **69**, 849-855.

Escoffier, C., Manie, S., Vincent, S., Muller, C. P., Billeter, M. & Gerlier, D. (1999). Nonstructural C protein is required for efficient measles virus replication in human peripheral blood cells. *J Virol* **73**, 1695-1698.

Garcin, D., Itoh, M. & Kolakofsky, D. (1997). A point mutation in the Sendai virus accessory C proteins attenuates virulence for mice, but not virus growth in cell culture. *Virology* **238**, 424-431.

Garcin, D., Latorre, P. & Kolakofsky, D. (1999). Sendai virus C proteins counteract the interferon-mediated induction of an antiviral state. *J Virol* **73**, 6559-6565.

Georges-Courbot, M. C., Contamin, H., Faure, C., Loth, P., Baize, S., Leyssen, P., Neyts, J. & Deubel, V. (2006). Poly(I)-poly(C12U) but not ribavirin prevents

death in a hamster model of Nipah virus infection. *Antimicrob Agents Chemother* **50**, 1768-1772.

Gotoh, B., Komatsu, T., Takeuchi, K. & Yokoo, J. (2001). Paramyxovirus accessory proteins as interferon antagonists. *Microbiol Immunol* **45**, 787-800.

Horikami, S. M., Curran, J., Kolakofsky, D. & Moyer, S. A. (1992). Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *J Virol* **66**, 4901-4908.

Horikami, S. M., Smallwood, S., Bankamp, B. & Moyer, S. A. (1994). An amino-proximal domain of the L protein binds to the P protein in the measles virus RNA polymerase complex. *Virology* **205**, 540-545.

Kulkarni, S., Volchkova, V., Basler, C. F., Palese, P., Volchkov, V. E. & Shaw, M. L. (2009). Nipah Virus Edits its P Gene at High Frequency to Express the V and W Proteins. *J Virol*.

Lane, T. E., Hardison, J. L. & Walsh, K. B. (2006). Functional diversity of chemokines and chemokine receptors in response to viral infection of the central nervous system. *Curr Top Microbiol Immunol* **303**, 1-27.

Lo, M. K., Harcourt, B. H., Mungall, B. A., Tamin, A., Peeples, M. E., Bellini, W. J. & Rota, P. A. (2009). Determination of the henipavirus phosphoprotein gene mRNA editing frequencies and detection of the C, V and W proteins of Nipah virus in virus-infected cells. *J Gen Virol* **90**, 398-404.

Lo, M. K. & Rota, P. A. (2008). The emergence of Nipah virus, a highly pathogenic paramyxovirus. *J Clin Virol*.

Melchjorsen, J., Jensen, S. B., Malmgaard, L., Rasmussen, S. B., Weber, F., Bowie, A. G., Matikainen, S. & Paludan, S. R. (2005). Activation of innate defense against a paramyxovirus is mediated by RIG-I and TLR7 and TLR8 in a cell-type-specific manner. *J Virol* **79**, 12944-12951.

Mungall, B. A., Middleton, D., Crameri, G., Halpin, K., Bingham, J., Eaton, B. T. & Broder, C. C. (2007). Vertical transmission and fetal replication of Nipah virus in an experimentally infected cat. *J Infect Dis* **196**, 812-816.

Nakatsu, Y., Takeda, M., Ohno, S., Koga, R. & Yanagi, Y. (2006). Translational inhibition and increased interferon induction in cells infected with C protein-deficient measles virus. *J Virol* **80**, 11861-11867.

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M. & Yanagi, Y. (2008). Measles virus circumvents the host interferon response by different actions of the C and V proteins. *J Virol* **82**, 8296-8306.

Pallister, J., Middleton, D., Crameri, G., Yamada, M., Klein, R., Hancock, T. J., Foord, A., Shiell, B., Michalski, W., Broder, C. C. & Wang, L. F. (2009). Chloroquine administration does not prevent Nipah virus infection and disease in ferrets. *J Virol*.

Parisien, J. P., Bamming, D., Komuro, A., Ramachandran, A., Rodriguez, J. J., Barber, G., Wojahn, R. D. & Horvath, C. M. (2009). A shared interface mediates paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2. *J Virol* **83**, 7252-7260.

Park, D. W., Baek, K., Lee, J. G., Park, Y. K., Kim, J. H., Kim, J. R. & Baek, S. H. (2007). Activation of toll-like receptor 4 modulates vascular endothelial growth

factor synthesis through prostacyclin-IP signaling. *Biochem Biophys Res Commun* **362**, 1090-1095.

Park, M. S., Shaw, M. L., Munoz-Jordan, J., Cros, J. F., Nakaya, T., Bouvier, N., Palese, P., Garcia-Sastre, A. & Basler, C. F. (2003). Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. *J Virol* **77**, 1501-1511.

Pegu, A., Qin, S., Fallert Junecko, B. A., Nisato, R. E., Pepper, M. S. & Reinhart, T. A. (2008). Human lymphatic endothelial cells express multiple functional TLRs. *J Immunol* **180**, 3399-3405.

Pelet, T., Curran, J. & Kolakofsky, D. (1991). The P gene of bovine parainfluenza virus 3 expresses all three reading frames from a single mRNA editing site. *Embo J* **10**, 443-448.

Plumet, S., Herschke, F., Bourhis, J. M., Valentin, H., Longhi, S. & Gerlier, D. (2007). Cytosolic 5'-triphosphate ended viral leader transcript of measles virus as activator of the RIG I-mediated interferon response. *PLoS One* **2**, e279.

Rodriguez, J. J., Cruz, C. D. & Horvath, C. M. (2004). Identification of the nuclear export signal and STAT-binding domains of the Nipah virus V protein reveals mechanisms underlying interferon evasion. *J Virol* **78**, 5358-5367.

Rodriguez, J. J., Parisien, J. P. & Horvath, C. M. (2002). Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. *J Virol* **76**, 11476-11483.

Schlee, M., Roth, A., Hornung, V., Hagmann, C. A., Wimmenauer, V., Barchet, W., Coch, C., Janke, M., Mihailovic, A., Wardle, G., Juranek, S., Kato, H., Kawai, T., Poeck, H., Fitzgerald, K. A., Takeuchi, O., Akira, S., Tuschl, T., Latz, E., Ludwig, J. & Hartmann, G. (2009). Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* **31**, 25-34.

Schmidt, A., Schwerd, T., Hamm, W., Hellmuth, J. C., Cui, S., Wenzel, M., Hoffmann, F. S., Michallet, M. C., Besch, R., Hopfner, K. P., Endres, S. & Rothenfusser, S. (2009). 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proc Natl Acad Sci U S A* **106**, 12067-12072.

Shaw, M. L., Cardenas, W. B., Zamarin, D., Palese, P. & Basler, C. F. (2005). Nuclear localization of the Nipah virus W protein allows for inhibition of both virus- and toll-like receptor 3-triggered signaling pathways. *J Virol* **79**, 6078-6088.

Shaw, M. L., Garcia-Sastre, A., Palese, P. & Basler, C. F. (2004). Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. *J Virol* **78**, 5633-5641.

Sleeman, K., Bankamp, B., Hummel, K. B., Lo, M. K., Bellini, W. J. & Rota, P. A. (2008). The C, V and W proteins of Nipah virus inhibit minigenome replication. *J Gen Virol* **89**, 1300-1308.

Sugahara, F., Uchiyama, T., Watanabe, H., Shimazu, Y., Kuwayama, M., Fujii, Y., Kiyotani, K., Adachi, A., Kohno, N., Yoshida, T. & Sakaguchi, T. (2004).

Paramyxovirus Sendai virus-like particle formation by expression of multiple viral proteins and acceleration of its release by C protein. *Virology* **325**, 1-10.

Torres-Velez, F. J., Shieh, W. J., Rollin, P. E., Morken, T., Brown, C., Ksiazek, T. G. & Zaki, S. R. (2008). Histopathologic and immunohistochemical characterization of Nipah virus infection in the guinea pig. *Vet Pathol* **45**, 576-585.

Weber, F., Wagner, V., Rasmussen, S. B., Hartmann, R. & Paludan, S. R. (2006). Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J Virol* **80**, 5059-5064.

Weingartl, H., Czub, S., Copps, J., Berhane, Y., Middleton, D., Marszal, P., Gren, J., Smith, G., Ganske, S., Manning, L. & Czub, M. (2005). Invasion of the central nervous system in a porcine host by nipah virus. *J Virol* **79**, 7528-7534.

Wong, K. T., Shieh, W. J., Kumar, S., Norain, K., Abdullah, W., Guarner, J., Goldsmith, C. S., Chua, K. B., Lam, S. K., Tan, C. T., Goh, K. J., Chong, H. T., Jusoh, R., Rollin, P. E., Ksiazek, T. G. & Zaki, S. R. (2002). Nipah virus infection: pathology and pathogenesis of an emerging paramyxoviral zoonosis. *Am J Pathol* **161**, 2153-2167.

Yoneda, M., Guillaume, V., Ikeda, F., Sakuma, Y., Sato, H., Wild, T. F. & Kai, C. (2006). Establishment of a Nipah virus rescue system. *Proc Natl Acad Sci U S A* **103**, 16508-16513.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr., Akira, S., Yonehara, S., Kato, A. & Fujita, T. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* **175**, 2851-2858.