The Characterization of Nipah virus V and W proteins

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1. Introduction

1.1 Nipah virus (NiV)

1.1.1 History

Nipah virus (NiV) and closely related Hendra virus (HeV) are members of the genus *Henipavirus* in the family *Paramyxoviridae*. NiV emerged as the cause of an outbreak of disease in pigs and in humans in Peninsular Malaysia in 1998 through 1999. A small number of cats, dogs and horses also became infected. In the commercial pig population of Peninsular Malaysia, a respiratory disease rapidly spread assisted by the movement and sale of pigs from one farm to another. It appears that pigs served as amplifying hosts, with all human patients infected through contact with infected pigs (Mohd Nor *et al.*, 2000). By June 1999, more than 265 human cases of encephalitis, including 105 deaths, had been reported in Malaysia and 11 cases of disease with one death had been reported in Singapore (Chua *et al.*, 1999; Paton *et al.*, 1999). Following the control of this outbreak by the slaughter of almost 1.2 million pigs, it was hoped that such an outbreak would never be seen again. However in 2001, an outbreak of acute encephalitis in Bangladesh was finally attributed to NiV infection. NiV has continued to re-emerge in Bangladesh and the bordering Indian State of Siliguri, causing fatal encephalitis and for the first time, person-to-person transmission appeared to have been a primary mode of spread (Butler, 2004; Chadha *et al.*, 2006; Enserink, 2004; Hsu *et al.*, 2004). The case mortality rate in the outbreak in Bangladesh was approximately 75%, which is significantly higher than any other NiV outbreak to date (ICDDR, 2005; Knipe D.M., 2007). NiV outbreaks have occurred in Bangladesh almost every year since the first

outbreak in 2001 (Knipe D.M., 2007). In addition, during these outbreaks it has not been shown that domestic or other animals became infected, indicating that perhaps for the first time there has been direct transmission of the virus from its reservoir host to humans. The emergence of henipavirus infections might not be restricted to the South-east Asian area since a recent study reported about the evidence of henipavirus infection in West African fruit bats (Hayman *et al.*, 2008). The susceptibility of humans, the virulence of the viruses and absence of therapeutics and vaccines lead to the classification of NiV and HeV as Biosafety Level 4 (BSL4) pathogens.

1.1.2 Reservoir hosts

For most paramyxoviruses, host range is limited and interspecies transmission is rare. One of theses rare interspecies transmission events is represented by the canine distemper virus which was found to infect a broad host spectrum of Carnivora such as lions, jaguars and seals (Harder & Osterhaus, 1997). Interestingly, henipaviruses display a broad species tropism (Knipe D.M., 2007). Serological surveillance and virus isolation studies have indicated that the reservoir host of NiV and HeV are flying foxes (bats) belonging to the genus Pteropus, family Pteropodidae (Chua *et al.*, 2002; Halpin *et al.*, 2000). Experimental infections of flying foxes with both NiV and HeV has shown that animals do not succumb to disease, and infrequently shed the virus, most often in urine. They are capable of mounting a humoral response and upon seroconversion low to moderate antibody titers are detectable (Middleton *et al.*, 2007). Future directions for research will focus on the reservoir host, to learn more about the bat's immune system and the ways in

which they deal with these and other viruses (Omatsu *et al.*, 2007). The emergence of these and related viruses is probably associated with the destruction of the flying fox native habitats, driving the animals to seek food from orchards and ornamental trees in urban and periurban areas (Daszak, 2006).

1.1.3 Molecular biology

The genome of NiV is 18 246 nucleotides in length and contains six transcription units encoding six viral structural proteins (3'-N-P-M-F-G-L-5') and three predicted non-structural proteins, C, V and W (Harcourt *et al.*, 2000) (Fig. 1). The number and order of henipavirus genes are identical to those found in respiroviruses and morbilliviruses. Six transcription units encode six major structural proteins namely the nucleocapsid (N), phosphoprotein (P), matrix (M) protein, glycoprotein (G) and large (L) protein or RNA polymerase (Knipe D.M., 2007). The genomic termini of NiV and HeV are highly conserved and complementary, as in other paramyxoviruses (Knipe D.M., 2007). Like all other viruses of the subfamily *Paramyxovirinae* NiV and HeV have genome lengths, which are multiple of six. Minireplicon assays have shown that NiV and HeV obey the "rule of six" (Halpin *et al.*, 2004; Knipe D.M., 2007). It has been proposed that the templates for transcription and replication are Ns in which each nucleoprotein subunit is associated with six nucleotides of genomic RNA. Genomes whose lengths deviate from the "rule of six" do not replicate efficiently (Calain & Roux, 1993). NiV and HeV have a high level of nucleotide and amino acid similarity. The amino acid sequence identity between the N, P and L proteins of NiV and HeV is 92%, 71% and 87%, respectively.

Overall, these viruses share a greater level of nucleotide similarity in their protein encoding regions (70-80%) than in the 5'- and 3'UTRs of each gene (40-67%). However, the genomic leader and trailer sequences have 80% and 90% similarity, respectively (Harcourt *et al.*, 2001).

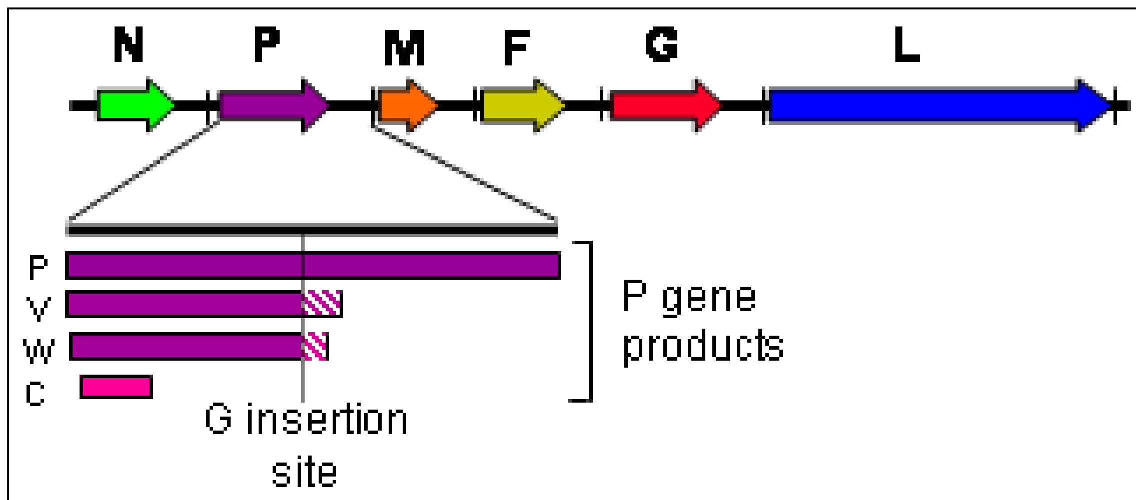


Fig. 1: The henipavirus genome.
The negative-sense genomic RNA is presented in the 3' to 5' orientation. The open reading frames indicated by the colored arrows encode the nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein or attachment protein (G) and large protein (L) or RNA polymerase, in the order 3'-N-P-M-F-G-L-5'. The vertical lines represent gene start and stop signals. Note the long untranslated 3' regions in all genes except the L gene. All genes except the P gene are monocistronic. The P gene of henipaviruses encodes not only for the P protein, but also V, W and C proteins. Genomic RNA in RNPs is transcribed by the viral polymerase, which associates with the RNP at the 3' terminus and sequentially generates discrete mRNAs from each of the viral genes. The mRNAs are not produced in equimolar amounts and there is a transcription gradient from the N to the L gene.

NiV P gene. All NiV genes except the P gene are monocistronic. The P gene of NiV is predicted to encode four proteins, namely P, V, W and C (Harcourt *et al.*, 2000; Wang *et*

al., 2001) (Fig. 1). As for other paramyxoviruses, the C protein of NiV is expressed from an alternative open reading frame (ORF) within the phosphoprotein (P) gene, whereas the V and W proteins are expressed by RNA editing (Knipe D.M., 2007). At a unique, highly conserved RNA-editing site in the P gene, the polymerase (L) inserts a single, non-templated G residue that results in a frame shift and the expression of the V protein. Insertion of two non-templated G residues results in expression of the W protein (Harcourt *et al.*, 2000). Whilst the C protein of NiV and other paramyxoviruses are unique and share no sequence similarity with the P protein, the V and W proteins share an amino-terminal 407 amino acids (aa) domain with P and each possesses a unique carboxyl-terminal domain consisting of 52 aa for V and 47 aa for W (Harcourt *et al.*, 2000). The sequences of the P proteins of paramyxoviruses are not well conserved (Baron *et al.*, 1993) and, whilst a V protein is not expressed by all paramyxoviruses, the unique C terminal domain of V is more conserved than the N terminus shared with P (Galinski *et al.*, 1992; Matsuoka *et al.*, 1991; Witko *et al.*, 2006). The V proteins of a number of paramyxoviruses evade host cell interferon (IFN) signal transduction and subsequent antiviral responses by inducing proteasomal degradation of the IFN-responsive transcription factors, STAT1 or STAT2 (Conzelmann, 2005; Garcia-Sastre, 2002; Horvath, 2004). While the target of the C protein is not known, it has been shown that NiV P, V and W proteins all act on the IFN signaling or JAK/STAT pathway by a distinct mechanism involving direct inhibition of STAT1, through a domain in their common N terminus (Rodriguez & Horvath, 2004; Shaw *et al.*, 2004). This is accomplished when the V protein forms high molecular weight complexes with both STAT1 and STAT2 (Rodriguez *et al.*, 2002; Rodriguez *et al.*, 2003). The V protein of the henipaviruses

accumulates in the cytoplasm, alters the STAT protein subcellular distribution in the steady state and prevents IFN-stimulated STAT redistribution. As a result the viral V protein efficiently inhibits STAT1 and STAT2 nuclear translocation in response to IFN. This leads to an inhibition of cellular responses to both IFN-alpha and IFN-gamma (Rodriguez *et al.*, 2002). The related W protein also functions as an inhibitor of JAK/STAT signaling. In addition NiV W contains a nuclear localization signal (NLS) within the unique C terminus, which enables it to inhibit the promotor activity in response to stimulation of Toll-like receptor (TLR3) by extracellular double-stranded RNA (dsRNA) (Shaw *et al.*, 2005). The henipavirus P gene is larger than any of its paramyxovirus counterparts, and the encoded P, V and W proteins have an N-terminal extension of approximately 100-200 amino acids compared with cognate proteins in the subfamily (Chua *et al.*, 2000a; Wang *et al.*, 2000). The minimum domain required for IFN-antagonist activity and STAT binding maps to this region, between amino acids 50-100 (Shaw *et al.*, 2004). The V protein of HeV has also been shown to target the viral sensor protein MDA5 (Andrejeva *et al.*, 2004). Whether the binding activity of the HeV V protein resides in the C-terminal, cysteine-rich terminal domain, like the respirovirus simian parainfluenzavirus 5 (SV5), or the N-terminal domain, the site of henipavirus anti-IFN-signaling activity, is not yet known.

1.1.4 Therapy

There are currently no vaccines or post-exposure therapeutics specifically indicated for henipavirus infection. A limited-non-randomized trial of ribavirin during the initial NiV outbreak in Malaysia showed ribavirin therapy was able to reduce mortality of acute NiV

encephalitits (Chong *et al.*, 2001). Ribavirin inhibits replication of HeV in vitro (Chong *et al.*, 2001) and a more recent study showed that the 5-ethyl analogue of ribavirin was able to prevent mortality in a hamster model of NiV infection (Georges-Courbot *et al.*, 2006). In the absence of other therapies, ribavirin is an option for treatment. There are also a number of therapy options being explored experimentally including active and passive vaccination (Guillaume *et al.*, 2004; McEachern *et al.*, 2008; Mungall *et al.*, 2006; Tamin *et al.*, 2002; Weingartl *et al.*, 2006; White *et al.*, 2005; Zhu *et al.*, 2008), fusion inhibition (Bossart *et al.*, 2005; Bossart *et al.*, 2001; Bossart *et al.*, 2002) and receptor blockade (Bonaparte *et al.*, 2005; Negrete *et al.*, 2005; Negrete *et al.*, 2006).

1.2 The interferon system

The innate immune system plays a critical role in detecting viral infections and evoking anti-viral responses and the host IFN system serves as one of the primary defense mechanisms (Basler & Garcia-Sastre, 2002). The IFN system can be divided into IFN induction and IFN signaling (Fig. 2). Initially infected non-immune cells as well as innate immune cells such as macrophages and dendritic cells (DCs) recognize viruses. These cells rapidly evoke anti-viral responses via the production of type I IFNs and proinflammatory cytokines. Type I interferons are comprised of 13 IFN-alpha isoforms and a single IFN-beta (Honda *et al.*, 2006; Stetson & Medzhitov, 2006). In addition to activating natural killer cells (NK) and T cells, IFNs and cytokines induce apoptosis of virus-infected cells and cellular resistance to viral infection. Thus, type I IFNs have essential roles in both innate and adaptive immune system.

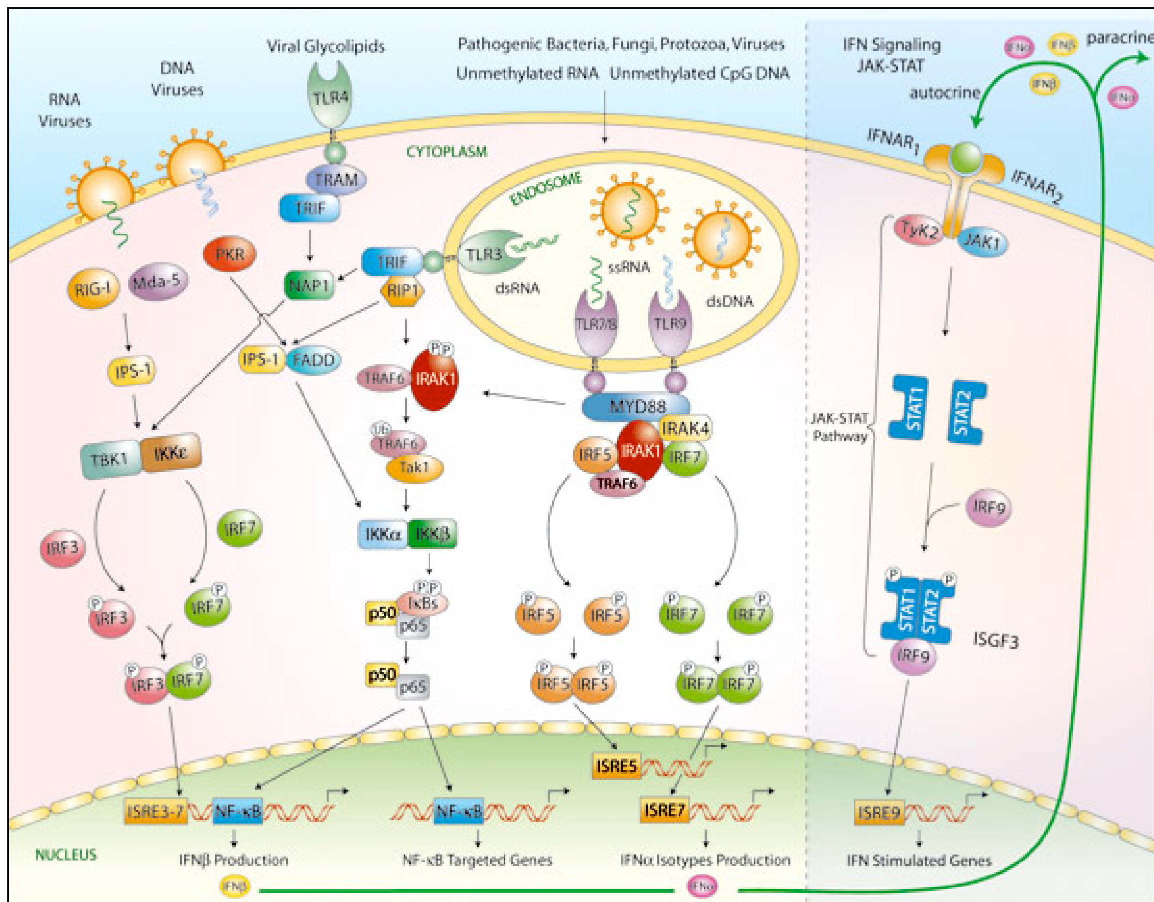


Fig. 2: Type I interferon production and signaling.
(kindly provided by InvivoGen).

Intracellular signaling cascades regulate the expression of type I IFNs and cytokine genes. Germ-line encoded pattern recognition receptors recognize molecular patterns that are specific to microorganisms such as lipopolysaccharide, bacterial lipoproteins, and nucleotides. Three classes of innate pattern recognition receptors have been identified:

TLRs, retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs), and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins.

During virus infection, replication intermediates such as double-stranded RNA (dsRNA) are produced. RIG-I is comprised of two N-terminal caspase recruitment domains (CARDs), followed by a DExD/H box helicase domain (Yoneyama *et al.*, 2004). RIG-I forms a family with melanoma differentiation-associated gene-5 (MDA5) and LGP2, based on their high similarities of their helicase domain (Kang *et al.*, 2002; Kovacsovics *et al.*, 2002; Yoneyama *et al.*, 2005). The expression of these proteins is strongly induced by IFNs, and they interact with dsRNA through their helicase domains. Besides the CARDs and the helicase domains, a C-terminal portion of RIG-I, namely the repressor domain (RD), was found to be important for controlling RIG-I-mediated IFN responses (Saito *et al.*, 2007). The functional roles of RIG-I and MDA5 in vivo have been investigated in mice deficient in these molecules (Gitlin *et al.*, 2006; Kato *et al.*, 2005; Kato *et al.*, 2006). Analyses with RIG-I$^{-/-}$ and MDA5$^{-/-}$ mice revealed that RIG-I and MDA5 recognize different RNA viruses. RIG-I is a sensor for various RNA viruses including paramyxoviruses, influenza virus, vesicular stomatitis virus (VSV), and the Japanese encephalitis virus (JEV). MDA5 seems to specifically recognize Picornaviruses such as encephalomyocarditis virus (EMCV), Theiler's virus and Mengo virus. Similarly to viruses, synthetic RNAs are also differentially recognized by RIG-I and MDA5. MDA5 has been shown to recognize polyinosinic polycytidylic acid (poly I:C) (Kato *et al.*, 2006). In contrast, RIG-I detects in vitro-transcribed dsRNA. A recent study reported that 5'-triphosphate ssRNA was able to stimulate IFN production by RIG-I but not MDA5 (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). Thus, RIG-I can discriminate

between 5'-triphosphorylated RNAs from some viruses and host mRNA whose ends are capped. However, also small dsRNAs (ranging from 21 to 27 nucleotides) without 3' overhangs have been shown to induce IFN-stimulated genes via RIG-I (Marques *et al.*, 2006). Small dsRNAs with 3' overhangs of two nucleotides, which can be a product of Dicer-mediated processing, fail to induce RIG-I helicase activity. Thus, it is still unclear whether the helicase activity of RIG-I is required for the recognition of RNAs and RNA viruses. On the other hand, the RNA structure that is responsible for MDA5 recognition has not been identified. LGP2 lacks a CARD motif and it has been suggested to function as a negative regulator of RIG-I/MDA5 signaling (Rothenfusser *et al.*, 2005; Yoneyama *et al.*, 2005). Overexpression of LGP2 inhibited Sendai virus and NDV signaling. LGP2 was reported to bind to dsRNA, thereby preventing RIG-I-mediated recognition. Moreover, LGP2 contains an RD, similar to the case for RIG-I (Alexopoulou *et al.*, 2001). The CARDs of both RIG-I and MDA5 are responsible for initiating signaling cascades. Binding to a previously identified CARD containing protein MAVS/IPS1/VISA/Cardif (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005) and via TRAF3, TBK1 and IKKi leads to the subsequent activation of transcription factors such as IRF-3 and NFkappaB and AP-1 family members, and as a consequence to IFN production and expression of IFN-stimulated response elements (ISREs) (Honda *et al.*, 2005; Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). Secreted IFNs signal through common receptors and activate a JAK/STAT signaling pathway. Upon receptor binding JAK kinases activate JAK1 and TYK2, which leads to the phosphorylation of STAT1 and STAT2. The phosphorylated STATs form heterodimers, associate with IRF9, and then translocate to the nucleus. This

complex is known as ISG factor 3 (ISGF3) and binds to ISREs within the promotor region of interferon-stimulated genes (ISGs). This results in the transcriptional upregulation of these genes and an antiviral state is established (Darnell, 1997).

Conventional dendritic cells (cDCs) and fibroblasts from RIG-I deficient mice were found to be almost completely impaired in the production of type I IFN in response to Sendai virus and NDV (Kato *et al.*, 2005). In contrast, plasmacytoic dendritic cells (pDCs), which are capable of producing copious amounts of IFN upon activation, appear to rely exclusively on the TLRs for viral recognition, indicating different cell-types utilize different viral sensors. TLRs are comprised of leucine-rich repeats (LRRs), a transmembrane domain, and a cytoplasmic domain designated the Toll/interleukin-1 receptor (IL-1R) homology TIR domain (Akira *et al.*, 2006; Beutler *et al.*, 2006). The microbial components recognized by each TLR have mostly been identified (Kawai & Akira, 2007). TLR3, TLR7 and TLR9 are localized on cytoplasmic vesicles, such as endosomes and endoplasmic reticulum (ER), and recognize microbial nucleotides. Specifically, TLR3 detects dsRNA, while TLR7 and TLR9 recognize ssRNA and DNA with CpG motif, respectively. TLR3 is also able to recognize dsRNA and poly I:C in macrophages. TLR3 activates the IFN-beta promotor as well as NFkappaB in response to poly I:C stimulation. Intracellular addition of poly I:C as well as virus infection of $TLR3^{-/-}$ cells resulted in the production of IFN. Upon stimulation with poly I:C, TLR3 triggers a signaling cascade via a TIR-domain-containing adapter inducing IFN-beta (TRIF) (Oshiumi *et al.*, 2003; Yamamoto *et al.*, 2002). TRIF is also responsible for IFN-beta production in response to bacterial LPS, the TLR4 ligand (Hoebe *et al.*, 2003; Yamamoto *et al.*, 2003). TRIF associates with TRAF3 and TRAF6. TRAF6 and RIP1 are able to

activate NFkappaB. In contrast, TRAF3 is responsible for inducing type I IFNs via the TRAF3-TBK1/IKKi pathway (Hacker *et al.*, 2006; Oganesyan *et al.*, 2006). pDCs produce high levels of TLR7 and TLR9, which recognize ssRNA and unmethylated DNA with CpG motifs, respectively (Diebold *et al.*, 2004; Heil *et al.*, 2004; Hemmi *et al.*, 2000). These TLRs recruit myeloid differentiation factor MyD88 (Hemmi *et al.*, 2002; Hemmi *et al.*, 2000) and activate IRAK-4, IRAK-1, and TRAF6, resulting in activation of the IKK complex and nuclear translocation of IRF7 and NFkappaB, which in turn initiates the expression of type I IFNs and pro-inflammatory cytokine genes (Hacker *et al.*, 2006; Honda *et al.*, 2004; Hoshino *et al.*, 2006; Kawai *et al.*, 2004; Oganesyan *et al.*, 2006; Uematsu *et al.*, 2005).

Viruses have developed several strategies in order to circumvent the IFN-induced antiviral state (Garcia-Sastre, 2001; Katze *et al.*, 2002; Levy & Garcia-Sastre, 2001). Among negative-stranded RNA viruses, several IFN antagonists have been identified, such as the influenza virus NS1 protein (Garcia-Sastre *et al.*, 1998), the Bunyamwera virus NSs proteins (Weber *et al.*, 2002) or the Ebola VP35 protein (Basler *et al.*, 2000). Moreover, there are numerous IFN antagonists within the paramyxoviruses (Gotoh *et al.*, 2002). The paramyxovirus IFN antagonist functions are all encoded by the phosphoprotein gene and are most predominantly carried out by one of the accessory V and C proteins. These proteins all target the IFN signaling or JAK/STAT pathway, but do so through distinct mechanisms. The Sendai virus C proteins interact with STAT1 and prevent pY701-STAT1 formation in response to IFN stimulation (Garcin *et al.*, 2001; Garcin *et al.*, 1999; Takeuchi *et al.*, 2001). There is also evidence that these proteins can

affect the dephosphorylation and stability of STAT1 and that the different C proteins may have differential effects (Garcin *et al.*, 2003; Garcin *et al.*, 2002; Saito *et al.*, 2002). The simian virus 5 (SV5) V protein targets STAT1 for degradation through the assembly of a degradation complex that also requires the presence of STAT2, damaged DNA binding protein 1, and cullin 4A (Andrejeva *et al.*, 2002; Didcock *et al.*, 1999b; Parisien *et al.*, 2002; Ulane *et al.*, 2003). The V protein of SV5 also inhibits IFN-beta synthesis suggesting that this single protein has the ability to target multiple pathways (He *et al.*, 2002; Poole *et al.*, 2002). The V protein of human parainfluenza virus 2 targets STAT2 for degradation through a mechanism analogous to that described for SV5 V (Nishio *et al.*, 2001; Parisien *et al.*, 2001; Ulane & Horvath, 2002; Young *et al.*, 2000). Similarly, the mumps virus V protein causes loss of STAT1 (Kubota *et al.*, 2001; Yokosawa *et al.*, 2002) as well as STAT3 (Ulane *et al.*, 2003). The V protein of measles virus, a morbillivirus, prevents nuclear translocation of STAT1 and STAT2 following IFN treatment but does not affect tyrosine phosphorylation (Palosaari *et al.*, 2003). The NDV V protein has also been shown to have IFN antagonist activity (Huang *et al.*, 2003; Park *et al.*, 2003b). There is also evidence that NDV V protein is able to induce the loss of STAT1 (Huang *et al.*, 2003). However, the P gene of Nipah virus is predicted to encode four proteins, namely P, V, W and C (Harcourt *et al.*, 2000; Wang *et al.*, 2001). All four proteins have been demonstrated to have IFN antagonistic activity (Park *et al.*, 2003b; Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). The target of the C protein is not known, but the P, V and W proteins act on the IFN signaling or JAK/STAT pathway. Henipaviruses inhibit IFN signaling by sequestering STAT proteins in high molecular weight complexes (Rodriguez *et al.*, 2002; Rodriguez *et al.*, 2003). This inhibition occurs via interaction

with STAT1 through a domain in the common N terminus of P, V and W protein, which prevents the phosphorylation and activation of STAT1 (Rodriguez *et al.*, 2004; Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). The anti-IFN signaling activity is a property of the V protein, as has been observed for other paramyxoviruses, but also for the W and P proteins (Rodriguez *et al.*, 2002; Rodriguez *et al.*, 2003; Shaw *et al.*, 2004). The P, V and W proteins of henipaviruses have an N terminal extension of aa 100 to 200 compared with cognate proteins of the subfamily (Chua *et al.*, 2000a; Wang *et al.*, 2000) and the STAT binding domain of NiV V maps to this region (Rodriguez *et al.*, 2004). The V and P proteins bind STAT in the cytoplasm, whereas the W protein, with its C terminal nuclear localization signal (NLS), co-localizes in the nucleus (Shaw *et al.*, 2005; Shaw *et al.*, 2004). NiV V protein has been described to shuttle between the cytoplasm and the nucleus and a nuclear export signal (NES) has been identified. This NES is required for its accumulation in the cytoplasm established via a Crm1-dependent nuclear export mechanism (Rodriguez *et al.*, 2004; Rodriguez *et al.*, 2002). It seems that Nipah virus has developed an advantageous strategy to target cellular factors within different cellular compartments. The W protein is the most and the P proteins the least efficient IFN antagonist (Park *et al.*, 2003b; Rodriguez *et al.*, 2004). The NiV C protein also displays a modest inhibition of IFN signaling but the mechanism and target are unknown (Park *et al.*, 2003b).

1.3 Dendritic cells

DCs constitute a unique system of cells because they represent a link between the innate and the adaptive immune response (Banchereau & Steinman, 1998; Palucka &

Banchereau, 1999). B- and T-lymphocytes are part of the adaptive immunity, but their function is under the control of dendritic cells. DCs are efficient stimulators of B and T lymphocytes. B cells, the precursors of antibody-secreting cells, can directly recognize native antigen through their B cell receptor. However, T cells need the antigen to be processed and presented to them by antigen-presenting cells (APCs). The T-cell antigen receptors (TCRs) recognize fragments of antigens bound to molecules of the major histocompatibility complex (MHC) on the surface of an APC. MHC class I stimulates cytotoxic T cells (CTLs) and MHC class II stimulates helper T cells. Intracellular antigens, processed in the cytoplasm of an APC, bind to MHC class I molecules and are recognized by CTLs. Activated CTLs are able to kill a target cell. Extracellular antigens that have entered the endocytic pathway of the APC are processed there and presented by MHC class II molecules to helper T cells. These specific cells have profound immune-regulatory effects. Located in most tissues, DCs capture and process antigens, and display large amounts of MHC-peptide complexes at their surface. Antigens are captured by DCs in peripheral tissues and processed to form MHC-peptide complexes. These immature DCs derive successfully from proliferating progenitors and non-proliferating precursors. As a consequence of antigen deposition and inflammation, DCs begin to mature. They express molecules that will lead to binding and stimulation of T cells in the T cell areas of lymphoid tissues. If B cells have also bound the antigen, then both B and T cells can cluster with DCs. After activation, T and B blasts leave the T cell area. B blasts move to the lining of the intestine, the bone marrow, and other parts of the lymphoid tissue. Some of them become antibody-secreting plasma cells. T blasts leave the blood at the original site of deposition, recognizing changes in the inflamed blood vessels and respond to

APCs. This limits the T cell response to the site of microbial infection. DCs display many fine dendrites that are long (>10 µm) and are either spiny or sheet-like. The shape and motility of DCs fit their functions, which are to capture antigens and select antigen-specific T cells. Mature DCs resist the suppressive effect of IL-10, but synthesize high levels of IL-12 (Cella *et al.*, 1997; Koch *et al.*, 1996; Reis e Sousa *et al.*, 1997) that enhance both innate (NK cells) and acquired (B and T cells) immunity. DCs also express many accessory molecules that interact with receptors on T cells (Caux *et al.*, 1994b; Inaba *et al.*, 1994) to enhance adhesion and signaling/co-stimulation, for example LFA-3/CD58, ICAM-1/CD54, B7-2/CD86. Depending on the conditions, DCs are able to stimulate the outgrowth and activation of a variety of T cells. They are able to persuade $CD8^+$ CTLs to proliferate vigorously (Bender *et al.*, 1995; Bhardwaj *et al.*, 1994). In the presence of mature DCs and of the IL-12 they produce, $CD4^+$ helper T cells turn into IFN-gamma producing Th1 cells. IFN-gamma activates the antimicrobial activities of macrophages and, together with IL-12, it promotes the differentiation of T cells into killer cells. However, in the presence of IL-4 DCs induce T cells to differentiate into Th2 cells which secrete IL-5 and IL-4. These cytokines activate eosinophils and help B cells to make the appropiate antibodies, respectively. The communication between DCs and T cells seem to be a dialogue rather than a monologue in which the DCs respond to T cells as well. CD40 (Caux *et al.*, 1994a) and TRANCE/RANK receptor (Anderson *et al.*, 1997; Wong *et al.*, 1997) on DCs are ligated by the tumor-necrosis factor (TNF) family of proteins that are expressed on activated and memory T cells. This leads to increased DC survival (Caux *et al.*, 1994a; Wong *et al.*, 1997) and, in the case of CD40, upregulation of CD80 and CD86 (Caux *et al.*, 1994a), secretion of IL-12 (Cella *et al.*, 1996; Koch *et al.*,

1996) and the release of chemokines such as IL-8, MIP1-alpha and MIP1-beta (Caux *et al.*, 1994a). In addition DCs contribute to the antiviral innate immune system by secreting IFN-alpha and IFN-beta in response to viral infection. During viral infection conventional DCs utilize RLHs as viral sensors, whereas plasmacytoid DCs rely on the TLR system (Takeuchi & Akira, 2007). Moreover, DCs not only activate lymphocytes, they also tolerate T cells to self-antigens in order to minimize autoimmune reactions. Given the importance of dendritic cells, there have been numerous approaches to exploit this cell type for DC-based immunotherapy to treat cancer and infectious diseases (Josien *et al.*, 1997; Shimizu *et al.*, 2007; Zitvogel *et al.*, 1998). Paradoxically, instead of inducing host resistance, DCs also provide a good environment for viruses and pathogens, in general. Cells of the DC system may be hosting latent cytomegalovirus (Soderberg-Naucler *et al.*, 1997), human immunodeficiency virus type I (Cavrois *et al.*, 2008), measles virus (de Witte *et al.*, 2008), respiratory syncytial virus (Munir, Le Nouen et al. 2008) or influenza virus A (Munir *et al.*, 2008; Wang *et al.*, 2008).

1.4 Questions and aims of this study

This study is about the characterization of the Nipah virus V and W proteins and can be divided into two parts.

The first part of the study comprises the analysis of NiV V and W proteins regarding their effect on the human host immune response during viral infection. Hereby the primary focus is to determine which host immune genes are downregulated or inhibited in their expression in the presence of either NiV V or W protein. In order to carry out the

infection experiments recombinant NDVs expressing the respective NiV proteins are used. These recombinant viruses are also characterized in this study.

The second part of the study is about the role of the NiV V and W protein in viral replication. The goal is to determine if NiV V or W protein may be able to reduce viral replication. In this case it is of interest if NiV V or W protein may interact with NiV N protein, an important component of the viral polymerase. Furthermore the region within NiV V or W protein that is responsible for interaction with NiV N protein, is identified.

2. Materials and Methods

2.1 Material

2.1.1 Devices and Material

Autoclave	Castle
Bacteria shaker	New Brunswick Scientific
Blot Filterpaper	Whatman
Cell counter	Bright Line Hausser Scientific
Cell centrifuge	Thermo Scientific
Computer	Apple Macintosh
Confocal microscope	Leika
Electrophorese chamber	Biorad
ELISA reader	Biotek
Fluorescence microscope	Leika
Freezers	REVCO
Heat block	VWR
Ice machine	Follett
Incubators	Thermo Scientific
Laboratory scale	Denver Instrument
Magnetic stirrer	Fisher
Microwave	Kenmore
Nitrocellulose membrane	GE Healthcare
pH meter	Corning

Photometer	Thermo Spectromic
Pipets	Falcon, Gibson
Precision scale	Metler AE200
Radiographic films	Kodak
Refrigerators	Frigidaire
Rotator	Nutator
Sequencer	Genetic Analyzer Applied Biosystem
Sterile tissue culture hood	The Baker Company
Tabletop centrifuge	Beckman Coulter; Eppendorf
Thermal cyclers	Perkin Elmer; ABI7900 HT
Ultracentrifuge	Sorvall (SW-28)
Vacuum centrifuge	Eppendorf
Vortexer	Vortex Genie 2 Scientific Industries
Water bath	Fisher

2.1.2 Radiochemicals

C^{14} Isotope	GE Healthcare

2.1.3 Enzymes

Restriction enzymes	BioLabs
DNA Ligase	Roche
Taq Polymerase	Taq platinium, Invitrogen

2.1.4 Kits

Absolutely RNA Miniprep Kit	RNA extraction	Stratagene
Beadlyte Human Multi-Cytokine Beadmaster Kit	ELISA	Upstate Cell Signaling
ECL Western Blot Detection Kit	Immunoblot development	Perker Elmer
Lipofectamine 2000	Transfection	Invitrogen
PCR Purification Kit	PCR purification	Qiagen
Plasmid Maxiprep Kit	Plasmid preparation	Qiagen
Plasmid Miniprep Kit	Plasmid preparation	Qiagen
QIAquick Gel Extraction Kit	Gel extraction and DNA purification	Qiagen
Rapid Ligation Kit	DNA ligation	Roche
VeriKine Human IFN-BETA ELISA Kit	ELISA	PBL

2.1.5 Length- and size standards

Nucleic acid length standard

1kB Plus DNA Ladder	Invitrogen

Protein size standard

SeeBlue Plus2 Pre-stained standard	Invitrogen
MagicMark XP Western Blot Standard	Invitrogen

2.1.6 Antibiotics

	Stock solution	**Working solution**
Penicillin-Streptomycin	10,000 Units of penicillin (base) and 10,000 µg of streptomycin (base)/ ml	100 Units of penicillin and 100 µg of streptomycin/ ml
Geniticin	50 mg/ml	0.4 µg/µl

2.1.7 Bacteria strains

E. coli DH5alpha	Invitrogen, Transformation of ligation reactions
E. coli XL-10 Gold	Stratagene, ultracompetent

2.1.8 Cells and cell lines

Cells

Human dendritic cells	Isolated from blood of the New York Blood Center

Cell lines

293T	293 cell line expressing the SV-40 T-antigen, (DuBridge *et al.*, 1987)
A549	Human lung epithelial cell line, ATCC #CCL-185
BSR T7	Baby hamster kidney cell line constitutively expressing the T7 polymerase, (obtained from M. Ciancanelli)
DF1 (CEF)	Chicken embryo fibroblast immortal cell line, ATCC #CRL-12203
HeLa cells	Cervical cancer immortal cell line, ATCC # CCL-2.2
Vero	African green monkey kidney cell line, ATCC #CCL-81

2.1.9 Antibodies

2.1.9.1 Primary antibodies

Mouse anti-actin	Monoclonal antibody	SIGMA
Mouse anti-MxA	Monoclonal antibody	Obtained from Georg Kochs
Mouse anti-Myc	Monoclonal antibody	SIGMA
Mouse anti-NDV HN 7B1	Monoclonal antibody	Mounts Sinai Hybridoma Shared Research Facility
Mouse anti-RIG-I	Monoclonal antibody	Obtained from Estanislao Nistal-Villan
Mouse anti-HA	Polyclonal antibody	SIGMA
Rabbit anti-NDV NP	Polyclonal antibody	Obtained from Larry Leung
Rabbit anti-NiV V	Polyclonal antibody	Obtained from Megan L. Shaw
Rabbit anti-NiV W	Polyclonal antibody	Obtained from Megan L. Shaw

2.1.9.1 Secondary antibodies

sheep anti-mouse IgG HRP	GE Healthcare
donkey anti-rabbit IgG HRP	GE Healthcare

Goat anti-mouse Alexa Fluor 488	Sigma
Goat anti-rabbit Alexa Fluor 594	Sigma

2.1.10 Plasmids

pCAGGS	Mammalian expression plasmid
pTM1	T7-driven E.coli expression plasmid

2.1.11 Buffers and solutions

2.1.11.1 Bacteria culture

5x LB+ Medium

LB Broth Base Lennox L	100 g (Invitrogen #12780-029: contains 50 g Pepton, 25 g yeast extract and 25 g NaCl)
alpha-D Glucose	5 g
H_2O	ad 1000 ml, autoclave

LB Agar

imMedia™ Amp Agar	1 pouch (Invitrogen SKU#Q601-20)
H_2O	ad 200 ml, microwave

2.1.11.2 Solutions for analysis and cloning of DNA

50x TAE	Tris pH 8.0	2M
	Concentrated acetic acid	5.71 %
	EDTA pH 8.0	50 mM
6x Sample buffer	Bromine phenol blue	0.25 %
	Xylene cyanol	0.25 %
	Glycerol	30 %
1 M Tris-HCl pH 7.5	Tris	1 M
	Adjust pH with concentrated HCl	

2.1.11.3 Buffers and solutions for protein-biochemical methods

Lysis buffer cell lysates (stored at 4ºC)	Tris-HCl pH 7.5	50 mM
	NACl	280 mM
	EDTA	0.2 mM
	EGTA	2 mM
	Glycerol	10 %
	Dithiothreitol	1 mM

	Sodium vanadate Na_3VO_3	0.1 mM (add directly before use)
	Protease inhibitor	1 tablet (Complete; Roche, add directly before use)
2x Sample buffer for protein samples	Tris-HCl pH 6.8	125 mM
	SDS	4 %
	Glycerol	20 %
	beta-Mercaptoethanol	10 %
	Bromine phenol blue	0.1 %
10x SDS-PAGE buffer	Glycine	144.1 g
	Tris	30.3 g
	Sodium dodecyl sulfate	10g
	H_2O	ad 1000 ml
Transfer buffer (Blot buffer)	Tris	1.52 g
	Glycine	7.21 g
	Methanol	100 ml
	H_2O	ad 500 ml

PBS Tween (PBST)	PBS with	
	Tween 20	0.5 %

2.1.11.4 Cell culture

MEM (Minimal Essential Medium)	MEM (9.6 g/l, Invitrogen #41500-83; 2.2 g/l $NaHCO_3$)	
	FCS (Hyclone)	5% - 10%
	Glutamine	0.01 %
	Penicillin/Streptomycin	120 m g/l
DMEM (Dulbecco's Modified Eagle Medium)	DMEM (High Glucose, Invitrogen #41966-29)	
	FCS (Hyclone)	1% - 10%
	Penicillin/Streptomycin	120 m g/l
RPMI	Invitrogen	
	FCS	10 %
	Penicillin	100 units/ml
	Streptomycin	100 µg/ml
	Human GMCSF (human	500 units/ml (Peprotech)

	granulocyte-macrophage colony-stimulating factor)	
	Human IL-4 (human interleukin-4)	1,000 units/ml (Peprotech)
Overlay medium MEM F12 (Invitrogen)	F-12 powdered medium	1 sachet
	Penicillin/Streptomycin	10 ml
	Glutamine	10 ml
	35 % BSA	6 ml
	HEPES buffer (1M)	10 ml
	7.5% NaHCO3	18 ml
	H_2O	ad 500 ml
Overlay medium MEM F12 (Invitrogen) for CEF/DF1 cells	Overlay medium	25 ml
	H_2O	8.5 ml
	7.5% NaHCO3	0.67 ml
	Mg_2SO_4	0.37 g
	2 % Oxoid agar	15 ml
	Trypsin	125 µl
	1 % Dextran	1 ml

Freezing medium	FCS with	
	DMSO (SIGMA #D2650)	10 %

2.1.11.5 Standard solutions and buffers

PBS	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	4.3 mM
	KH_2PO_4	1.4 mM
	$CaCl_2$	1.5 mM
	$MgCl_2$	1 mM
PBS-	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	4.3 mM
	KH_2PO_4	1.4 mM
TBS	Tris-HCl	25 mM
	KCl	1 mM
	NaCl	150 mM

TE pH 8.0	Tris-HCl pH 8.0	10 mM
	EDTA pH 8.0	1 mM

2.2 Methods

2.2.1 Molecular-biological methods

2.2.1.1 DNA agarose gel electrophoresis

(Buffers and solutions: 2.1.11.2)

Agarose gel electrophoresis is a method used in biochemistry and molecular biology to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate farther than longer ones. Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules. High-percentage gels (2 %) were used for very small fragments and low-percentage gels (0.5-1%) were used for big fragments. The agarose was cooked in TAE buffer until it was completely dissolved (~ 60ºC). Before pouring the gel ethidium bromide (2 µl/100ml of a 10 mg/ml stock solution) was added. The DNA samples, mixed with 1x DNA sample buffer, were loaded onto the gel in a chamber that was filled with TAE. Then voltage was applied (20-200 V). Because of its

negative charge the DNA runs into the direction of the cathode. The DNA is visible under UV light because the stain ethidium bromide intercalates with the DNA and fluoresces.

2.2.1.2 Isolation of DNA fragments from agarose gels

After electrophoresis the gel is illuminated with an ultraviolet lamp (usually by placing it on a light box, while using protective gear to limit exposure to ultraviolet radiation) to view the DNA. The ethidium bromide fluoresces reddish-orange in the presence of DNA. The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA. Purification and elution was performed using the Qiagen Gel Extraction Kit.

2.2.1.3 Spectrometric determination of DNA concentration

In order to determine the DNA concentration the extinction of a watery DNA solution is measured at a wavelength of 260 nm (wavelength, at which light is absorbed the most by DNA). One OD unit equals 50 µg/ml dsDNA, 40 µg/ml ssDNA or 35 µg/ml RNA. In addition the extinction is measured at a wavelength of 280 nm. This is for determination of the purity level since also other molecules absorb at 260 nm (e.g. phenol, amino acids with aromatic side chains). The quotient of OD260 and OD280 gives information about the degree of purity. Clean DNA preparations should have a quotient between 1.7 and 2.0. Values above or below indicate contamination.

2.2.1.4 Restriction digestion of DNA

Restriction endonucleases cut double-stranded DNA in a sequence-specific manner and each restriction enzyme works best in a specific buffer. Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA. While recognition sequences vary widely, with lengths between 4 and 8 nucleotides, many of them are palindromic. Recognition sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation (5' end or the 3' end) of a sticky-end "overhang" of an enzyme restriction. If both strands are cut at the same place, there are no overhangs (blunt ends). Restriction digestions were used for the analysis of DNA.

Reaction

DNA	0.2 – 1 µg
10x buffer	2 µl
Enzyme	5 U
BSA	Depending on manufacturer's instructions
H_2O	ad 20 µl

One enzyme unit (1U) is defined as the amount of enzyme necessary to digest a µg lambda-DNA under optimal conditions in 1 h.

The digestion was incubated for 1.5 h to overnight at the temperature according to the manufacturer's protocol.

2.2.1.5 Dephosphorylation of DNA fragments

In order to circumvent the religation of DNA fragments after restriction digestion in a ligation reaction, the 5' ends of the linearized vector fragment are dephosphorylated by the enzym alkaline phosphatase (CIAP, "calf intestine alkaline phosphatase"). After the restriction digestion 10 U of the enzyme was added for another hour at 37ºC. Alkaline phosphatase is active in most restriction buffers.

2.2.1.6 Precipitation of DNA

In order to concentrate or desalt DNA solutions, DNA is precipitated with ethanol or isopropanol. Hereby, ethanol or isopropanol (in connection with elevated salt concentration) deprives DNA molecules of their hydrate envelope resulting in their aggregation and precipitation. After adding ethanol or isopropanol everything was mixed well and centrifuged for at least 15 min according to the Qiagen Maxiprep/Miniprep Kit. The supernatant was discarded, the pellet washed once with 70 % ethanol, dried and eluted in elution buffer of H_2O.

2.2.1.7 Ligation of linearized DNA fragments

The mechanism of DNA ligase is to form two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. ATP is required for the ligase reaction. For this reaction the DNA ends must have either complementary single-stranded overhangs (sticky ends) or no overhangs (blunt ends). The ligation was performed according to the manufacturer's instructions of the Rapid Ligation Kit.

2.2.1.8 Sequencing of DNA

DNA samples were sent to either Genewiz or the Mount Sinai Sequencing Facility for sequencing.

2.2.1.9 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by *in vitro* enzymatic replication. As PCR progresses, the DNA thus generated is itself used as a template for replication. This sets in motion a chain reaction in which the DNA template is exponentially amplified. With PCR it is possible to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of the DNA piece. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermus aquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary to physically separate the strands (at high temperatures) in a DNA double helix (DNA melting) used as template during DNA synthesis (at lower temperatures) by the DNA polymerase to selectively amplify the target DNA. The

selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

Reaction

Plasmid DNA or genetic DNA	200/500 ng
Primer sense	30-50 pMol
Primer antisense	30-50 pMol
10x buffer	5 µl
10 mM dNTP Mix (Invitrogen)	1 µl
Polymerase	2.5 U (Taq Polymerase)
H_2O	ad 50 µl

The reactions were set up on ice in order to avoid false primer binding (missprime). The reactions were preformed in a thermocyler (Perkin Elmer). In order to circumvent missprimethe reactions were put from the ice directly into the 95ºC-heated cycler.

PCR program for plasmid DNA as matrix

95ºC	30 sec	Denaturation
Hybridization temperature	40 sec	Hybridization
72ºC		Elongation

Total of 25 cycles

PCR program for genomic DNA as matrix

95ºC	2 min	Denaturation
95ºC	30 sec	Denaturation
Hybridization temperature	40 sec	Hybridization
72ºC		Elongation
Total of 35 cycles; the first denaturation step was only once performed		

Subsequently PCR products were purified using the Qiagen Kit for PCR product purification.

2.2.1.10 Transformation of plasmids in E. Coli bacteria

(Buffers and solutions: 2.1.11.1)

Competent bacteria take up plasmids during transformation. Transformation mostly serves for plasmid multiplication or for prokaryotic protein expression. Hereby one can distinguish between chemical transformation and electroporation. During the chemical transformation bacteria, rendered competent for plasmid up-take via a chemical treatment, take up the plasmid during a short heat shock period. On the other hand during electroporation bacteria receive en electrical pulse leading to the up-take of the plasmid through pores in the cell wall. In this study only chemical transformation was used.

- Bacteria are defrosted on ice
- 20-200 ng of DNA is added to 100 µl of bacteria suspension and mixed carefully
- incubation on ice for 30 min
- incubation at 42ºC for 30 sec
- incubation on ice for 2-3 min, add 500 µl of LB medium
- incubation at 37ºC for 1 h (shaker), then centrifuge at 13,000 rpm (table top centrifuge)
- discard supernatant, resuspend pellet in remaining media
- plate bacteria on LB-agar plates

Quicktransformation was used for transformation of already prepared plasmid DNA in bacteria (Pope & Kent, 1996). This high efficiency transformation serves to grow up larger amounts of plasmid DNA.

- Bacteria are defrosted on ice
- 0.5 µg plasmid of DNA is added to 50 µl of bacteria suspension and mixed carefully
- incubation on ice for 5 min
- plate bacteria on LB-agar plates (pre-heated to 37ºC)

2.2.1.11 Plasmid preparation from E. Coli via alkaline lysis

(Buffers and solutions: 2.1.11.1 and 2.1.11.2)

The preparation of small (Miniprep) amounts of plasmid DNA for analytical purposes and the preparation of large (Maxiprep) amounts of plasmid DNA for preparative purposes was performed according to the principle of the alkaline lysis (Birnboim & Doly, 1979). Hereby after cenrifugation of the bacteria suspension, the pellet is resuspended in RNAse A-containing buffer. The bacteria are lysed with a NaOH/SDS-containing solution. The basic condition leads to the denaturation of the bacterial proteins, the chromosomal DNA and the plasmid DNA. A subsequent neutralization with 3 M potassium acetate solution results in the precipitation of bacterial proteins and cell debris with the chromosomal DNA. However, the plasmid DNA stays soluble in a renatured form and therefore can be isolated. The bacterial RNA is digested via the added RNAse A and stays soluble after a precipitation step with isopropanol. Isopropanol does not precipitate RNA very efficiently under these conditions.

Maxi- and Minipreps were performed according to the manufacturer's instructions of the Maxi- or Miniprep Kit, respectively.

2.2.1.12 Cloning of expression plasmids

The NiV minigenome plasmid was made by Mike Ciancanelli based on previously published studies (Halpin *et al.*, 2004). Briefly, pSL1180 NiV GFP-CAT plasmid produces a NiV minigenome RNA from a T7 promotor. Hereby NiV genomic leader and trailer sequences flank a reporter gene encoding a green fluorescent protein-chloramphenicol-acetyltransferase (GFP-CAT) fusion protein. Leader and trailer sequences

correspond to GenBank accession number NC_002728 and were constructed by template-free PCR using overlapping deoxyoligonucleotides as templates. Adjacent to the leader sequence were placed the hepatitis delta virus ribozyme and T7 terminator sequence. In order to accomplish the rule of six, the minigenome length was made to be divisible by six by adding nucleotides between the GFP-CAT and the L non-coding region. The three fragments were assembled into the pSL1180 vector.

Constructs for co-immunoprecipitation experiments were cloned into the mammalian expression vector, pCAGGS (Niwa *et al.*, 1991). Cloning of the respective NiV plasmids being used was described elsewhere (Park *et al.*, 2003b; Shaw *et al.*, 2005; Shaw *et al.*, 2004).

2.2.1.13 Synthesis of recombinant Newcastle disease viruses

Recombinant NDV V(-) has been described previously (Park *et al.*, 2003a). The P, V and W genes of Nipah virus were inserted into Xba I site between P and M genes of NDV V(-) cDNA clone. Each gene was amplified by PCR with restriction enzyme site tagged primers: P forward 5'-CG**GCTAGC-**TTAGAAAAAA***T***ACGGGTAGAA*CACTAGTCCGCCACC*atggataaattggaactagtcaac; P reverse 5'-CGGCTAGCTCAAATATTACCGTCAATGATGTC; V and W forward 5'-GC**TCTAGA**TTAGAAAAAA***T***ACGGGTAGAA*TAGTCCGCCACC*atggataaattggaactagtcaac; V reverse 5'- GC**TCTAGA**TTAACCGCAGTGGAAGCATTC; W reverse 5'-GC**TCTAGA**CTAGTTGGACATTCTCCGCATTG (XbaI and NheI are in bold; the NDV gene start and gene end are underlined; The sequence of P, V, and W are in lowercase. One intergenic nucleotide T is in bold italics. The additional nucleotides are in

italics, which are able to follow the "rule of six" in the NDV genome). The plasmids pCAGGS-NiV P, V, W were used as templates for PCR amplification (Park *et al.*, 2003b; Shaw *et al.*, 2004). The PCR products and NDV V(-) vector were digested with either XbaI or NheI. After purification by PCR Purification kit (QIAGEN), the fragments and vector were ligated by Rapid Ligation Kit (Roche). All constructs were confirmed by sequencing.

The rescue of recombinant NDV V(-)-P, -V and -W viruses was following the procedures described previously (Nakaya *et al.*, 2001; Park *et al.*, 2003a).

2.2.2 Cell-biological and protein-biochemical methods

2.2.2.1 Cultivation of adherent cells

(Buffers and solutions: 2.1.11.4)

All in this study used cells were kept in a 37ºC incubator (5 % CO_2). The following media were used for the respective cell lines (PS = Penicillin-Streptomycin):

293 T	DMEM (Invitrogen); 10 % FCS, 1 % PS
A549	DMEM (Invitrogen); 10 % FCS, 1 % PS
BSR T7	DMEM (Invitrogen); 10 % FCS, 1 % PS, 0.4 µg /µl Geniticin
CEF/DF1	DMEM (Invitrogen); 10 % FCS, 0.15 %

	$NaHCO_3$)
HeLa	DMEM (Invitrogen); 10 % FCS, 1 % PS
Vero	DMEM (Invitrogen); 10 % FCS, 1 % PS

After reaching a confluence level of 70-90 % cells were washed with PBS, trypsinized and resuspended in cell culture medium (dilution 1:5 to 1:10) in order to continue cultivation.

2.2.2.2 Cultivation of human dendritic cells

(Buffers and solutions: 2.1.11.4)

Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation (Histopaque; Sigma Aldrich) from buffy coats of healthy human donors (New York Blood Center). $CD14^+$ cells were immunomagnetically purified using anti-human CD14 antibody-labeled magnetic beads and iron-based Midimacs LS columns (Miltenyi Biotec). After elution from the columns, cells were plated (0.5×10^6 cells/ml) in DC medium (RPMI [Invitrogen], 10% fetal calf serum [HyClone], 100 units/ml of penicillin, and 100 µg/ml streptomycin [Invitrogen]) supplemented with 500 U/ml human granulocyte-macrophage colony-stimulating factor (Peprotech) and 1,000 U/ml human interleukin-4 (IL-4, Peprotech) and incubated for 5 to 6 days at 37ºC.

2.2.2.3 Freezing and unfreezing of cells

(Buffers and solutions: 2.1.11.4)

In order to freeze cells, cells were trypsinized from the bottom of the tissue culture plate and pelleted via centrifugation at 1200 rpm for 5 min. The cells were resuspended in the required amount of FCS with 10 % DMSO and 1 ml aliquots were frozen at -80ºC.

For unfreezing the frozen cells were defrosted in a 37ºC water bath and immediately washed in 10 ml pre-warmed cell culture medium. After washing the cells were resuspended in fresh medium and seeded into new cell culture plates.

2.2.2.4 Transfection

During a transfection plasmid DNA is transferred into cells in order to achieve a transient gene expression. In this study transfections were carried out in suspension using the transfection agent Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. This transfection reagent is not toxic and did not have to be removed from the cells after transfection.

2.2.2.5 Preparation of cell lysates for protein gel electrophoresis

(Buffers and solutions: 2.1.11.3)

For protein electrophoresis cells are lysed and the proteins denatured. Hereby, Triton X-100 permeabilizes the membranes and SDS and beta-mercapto ethanol denature the proteins.

In order to prepare cell lysates cells were washed 1x with PBS. Then 500 µl of lysis buffer (containing proteinase inhibitors) was added and incubated on ice for 20 min (on shaker). Then cells were scraped off the tissue culture plate and the lysates were transferred into eppendorf tubes. After centrifugation in a tabletop centrifuge (15 min, 13,000 rpm, 4ºC), the supernatant was saved, mixed with 2x protein sample buffer and boiled at 95ºC for 5 min.

Cell lysates were stored at -80ºC.

2.2.2.6 Tricine-SDS-Polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

(Buffers and solutions: 2.1.11.3)

With the Tricine-SDS-PAGE (modified from (Schagger & von Jagow, 1987) protein samples can be separated according to their size in a 5 %- 15 % acrylamide gel. The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

SDS binds in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-2.2 g SDS/g protein), giving an approximately uniform

mass:charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

The degree of separation (10-220 kDa) depends on the amount of present acrylamide and the crosslinks between acrylamide and bisacrylamide. The acrylamide polymerizes via free radicals, which are released by APS (ammonium peroxysulfate) and stabilized by TEMED (N,N,N',N' – tetramethylenediamine), Acrylamide fibers are crosslinked by polymerized bisacrylamide. There are two layers of gel, namely stacking or spacer gel, and resolving or separating gel.

The stacking gel is a large pore polyacrylamide gel (4%T). This gel is prepared with Tris/HCl buffer pH 6.8 of about 2 pH units lower than that of electrophoresis buffer (Tris/Glycine). These conditions provide an environment for Kohlrausch reactions, as a result, proteins are concentrated to several fold and a thin starting zone of the order of 19 μm is achieved in a few minutes. This gel is cast over the resolving gel. The height of the stacking gel region is always maintained more than double the height and the volume of the sample to be applied. The resolving gel is a small pore polyacrylamide gel (3 - 30% acrylamide monomer) typically made using a pH 8.8 Tris/HCl buffer. In the resolving gel, macromolecules separate according to their size. Resolving gels have an optimal range of separation that is based on the percent of monomer present in the polymerization reaction; for example an 8%, 10% and 12% resolving gel can effectively used for separating proteins between, 24 – 205 kDa, 14-205 kDa, and 14-66 kDa proteins, respectively.

For this study ready-prepared gels (BIO-RAD) were used. Ususally 15 µl of samples were loaded onto the gel. The gel was run at 90-110 mA for 1-2 h.

2.2.2.7 Co-Immunoprecipitation

(Buffers and solutions: 2.1.11.3 and 2.1.11.5)

Immunoprecipitation of intact protein complexes is known as co-immunoprecipitation (Co-IP). Co-IP works by selecting an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. By targeting this *known* member with an antibody it may become possible to pull the entire protein complex out of solution and thereby identify unknown members of the complex.

For co-immunoprecipitation of NiV N, 293T cells were transfected with 2 µg of HA-tagged Nipah virus or influenza virus expression plasmids or an empty vector and 2 µg of Myc-tagged NiV N expression plasmid. Following a 24-hour incubation, cells were washed in ice-cold PBS and lysed in 500 µl of extract buffer for 30 min on ice. Extracts were centrifuged at 13,000 rpm for 15 min in an Eppendorf Centrifuge (tabletop centrifuge), the supernatant was collected, and 2 µl of anti-Myc antibody (Sigma) was added and incubated overnight at 4ºC with rotation. Protein A-agarose (Roche) was added, and incubated at 4ºC was continued for 1 to 2 h. The agarose was washed three times with extract buffer and boiled in 2X SDS-PAGE sample buffer. Proteins were analyzed by SDS-PAGE and immunoblotting with an antibody against Myc- or HA epitope as described before. 293T cells were used for these assays so as to achieve the highest possible transfection efficiency.

2.2.2.8 Western Blot

(Buffers and solutions: 2.1.11.3 and 2.1.11.5)

After protein samples were separated via Tricine-SDS-PAGE the Western Blot allows their transfer onto a (nitrocellulose) membrane. For this study the Western Blots were performed using the "Semi-fry procedure". Proteins on the membrane can be detected with specific antibodies; this is an indirect detection. First the protein is detected with a specific antibody. Then a secondary antibody binds to the F_C region of the primary antibody. The secondary antibody is linked to a peroxidase (in this study: HRP, horseradish peroxidase) which when exposed to an appropriate substrate drives a colorimetric reaction and produces a color. The light emission is detected via exposure to a radiographic film.

Western Blot

Setup:

Cathode plate (wet with blot buffer)

2 layers of blot filter paper (soaked in blot buffer)

acrylamide gel

nitrocellulose membrane (soaked in blot buffer)

2 layers of blot filter paper (soaked in blot buffer)

anode plate (wet with blot buffer)

> Blot for 1 h at 100 mA

Immunoblot

- Wash blot with PBS
- Block blot in PBS with 5 % skim milk powder for 1 h (on shaker)
- Wash blot briefly with PBS
- Incubate blot with primary antibody (in PBS with 5 % skim milk powder) for 2 h at RT or overnight at 4ºC
- Wash blot 3x briefly with PBST, 2x 10 min with PBST
- Incubate blot with secondary antibody (in PBS with 5 % skim milk powder) for 1 h at RT
- Wash blot 3x briefly with PBST, 4x 10 min with PBST

Detection

- Wash blot briefly with PBS
- Add 3 ml ECL for 3-5 min
- Place blot in plastic wrap, expose radiographic film for 1 sec to 1 min

2.2.2.9 Replication assay and CAT assay

Chloramphenicol Acetyl Transferase (CAT) is a bacterial enzyme, which inactivates chloramphenicol by acetylating it. It is used as a reporter gene for promoter studies.

For this study BSR T7 cells were transfected with the desired plasmid constructs and Firefly luciferase expression plasmid (under T7 promotor) in Opti-MEM medium

(Invitrogen) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Total amounts of transfected DNA were kept constant by including additional empty-vector plasmid DNA where applicable. Details of the NiV minigenome replication assay have been described elsewhere (Halpin *et al.*, 2004). After a 24-hour incubation cells were lysed in 100 µl of reporter lysis buffer (Promega). 45 µl of each sample was incubated with 78 µl of Tris [7.5], 1 µl of acetyl coenzyme A and 1 µl of radioactive ^{14}C-isotope for 2 hours in a 37ºC water bath. Then samples were subjected to acetone extraction and vacuum centrifugation. After elution in 20 µl of acetone, samples were applied to a TCL plate and subjected to chromatography in a chamber filled with chloroform and methanol for 15 min. TCL plate was placed in a phosphor screen cassette (Molecular Dynamics) overnight and phosphorescence was read by Typhoon phosphor image reader (GE Healthcare). Values were normalized to luciferase activity.

2.2.2.10 Luciferase assay

In biological research, luciferase commonly is used as a reporter to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest.

For this study transfected cells were harvested as described for the CAT assay (2.2.2.9). A fraction of the cell supernatant was mixed with Firefly Substrate (Promega) and bioluminescence was measured using a photometer.

2.2.2.11 RNA extraction

Samples of mock-infected and rNDV-infected cells (1 x 10^6 cells) were pelleted and RNAs were isolated and treated with DNAse by using an Absolutely RNA RT-PCR miniprep kit (Stratagene). RNAs were quantified using a Nanodrop spectrophotometer (Nanodrop Technologies).

2.2.2.12 Immunofluorescence

(Buffers and solutions: 2.1.11.3 and 2.1.11.5)

Immunofluorescence is the labeling of antibodies or antigens with fluorescent dyes. This technique is often used to visualize the subcellular distribution of biomolecules of interest. Immunofluorescent-labeled tissue sections or cultures are studied using a fluorescence microscope or by confocal microscopy.

For this study detection of proteins via immunofluorescence was used for both transfected and infected cells. Coverslips were placed into tissue culture plates during transfection/ infection.

- Remove medium from cells and fix cells with ice-cold methanol (20 min at -20ºC)
- Remove coverslips and place cell-side up on a piece of parafilm; wash each coverslip with PBST to rehydrate cells
- Block for 30 min at RT with 1% BSA in PBST
- Wash 2x 5 min with PBST; add primary antibody in the block solution (volume of 50-100 µl per coverslip) and incubate overnight at 4ºC

- Wash 4x 5 min with PBST; add secondary antibody (in block solution) and incubate for 1-2 h at RT in the dark
- Wash 4x 5 min
- Place a coverslip on a drop of vector mounting fluid (Vectashield; 5-10 µl) onto a slide and seal the coverslips with nail varnish
- Coverslips are stored at 4ºC in the dark

For immunofluorescence in A549 cells: A549 cells were seeded on coverslips 24 hours prior infection. Infection with rNDVs was carried out at a multiplicity (MOI) of 0.5. After a 22-hour incubation time cells were fixed and later on analyzed by fluorescence microscopy.

For immunofluorescence in HeLa cells: HeLa cells were transfected in suspension with 2 µg of the HA-tagged Nipah virus expression plasmids and 2 µg of the Myc-tagged NiV N expression plasmid and were seeded onto glass coverslips. Following a 24-hour incubation, they were fixed and later on analyzed by confocal fluorescence microscopy (Leika). HeLa cells were used, because they allow easy distinction of the cytoplasm and nuclear components.

2.2.2.13 Capture Enzyme-Linked ImmunoSorbent Assay (ELISA)

Enzyme-Linked ImmunoSorbent Assay (ELISA) is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample**.** ELISAs are simply a binding event between an antibody and an antigen with an enzymatic

detection method. They can be used to detect proteins, peptides, lipids and any antigen that can be recognized by an antibody. It can be built in numerous ways to maximize the sensitivity and specificity of an assay. Assays can be direct, indirect and even applied as sandwich assays.

As a principle in ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal. Thus in the case of fluorescence ELISA, when light of the appropriate wavelength is shone upon the sample, any antigen/antibody complexes will fluoresce so that the amount of antigen in the sample can be inferred through the magnitude of the fluorescence.

For this study capture enzyme-linked immunorsorbent assays (ELISA) were performed as multiplex assay according to the manufacturer's instructions to quantify the cytokines and chemokines in the DC supernatants. Plates were read in a Luminex plate reader. IFN-beta was not part of the multiplex ELISA and amounts were measured in a separate Sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol [from PBL]. Plates were read in an ELISA reader from Biotek Instruments.

2.2.2.14 Quantitative Real-Time PCR (qRT-PCR)

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR) or kinetic polymerase chain reaction, is a laboratory technique based on the polymerase chain reaction, which is used to amplify

and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in *real time* after each amplification cycle. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA.

Frequently, real-time polymerase chain reaction is combined with reverse transcription polymerase chain reaction to quantify low abundance messenger RNA (mRNA), enabling a researcher to quantify relative gene expression at a particular time, or in a particular cell or tissue type.

For this study qRT-PCR was performed using a previously published SYBR green protocol with an ABI7900 HT thermal cycler (Yuen *et al.*, 2002). Each transcript in each sample was assayed two times, and the mean cycle threshold was used to calculate the x-fold change and control changes for each gene. Three housekeeping genes were used for global normalization in each experiment (actin, rsp-11 and tubulin genes). Data validity by modeling of reaction efficiencies and analysis of measurement precision was determined as described previously (Yuen *et al.*, 2002).

2.2.2.15 Topic-defined PIQOR Immunology Microarray

A DNA microarray is a multiplex technology used in molecular biology and in medicine.

It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles of a specific DNA sequence. This can be a short section of a gene or other DNA element that are used as probes to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target.

In standard microarrays, the probes are attached to a solid surface by a covalent bond to a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others). The solid surface can be glass or a silicon chip, in which case they are commonly known as "gene chip" or colloquially "Affy chip" when an Affymetrix chip is used. Other microarray platforms, such as Illumina, use microscopic beads, instead of the large solid support. DNA arrays are different from other types of microarray only in that they either measure DNA or use DNA as part of its detection system.

For this study A549 cells were infected with rNDVs or mock infected for 18 hours at an MOI of 2. Samples were shipped to Miltenyi Biotech, and all the procedures for microarrays were performed according to their standard protocols. Briefly, RNAs were extracted from the cells and amplified. Fluorescence-labeled probes were hybridized to topic-defined PIQOR immunology arrays (human antisense) and subjected to overnight hybridization using a hybridization station. 1,070 immune genes were tested. The threshold for significantly upregulated genes was a ≥ 1.7 fold induction compared to baseline gene expression (obtained by mock infected samples). For further analysis by NDV V significantly induced genes were set as 100% and compared to values obtained by NiV V and W. A downregulation of ≥ 50% by NiV V and W was considered to be significant. This allowed for narrowing down the number to 39 genes. In order to examine whether NiV

V or W might have a stronger inhibitory effect on transcription of these genes, both values (for NiV V or W) were compared to each other. A ≥ 1.7 fold difference was considered as significant. This value was applied in order to be consistent with the threshold used by Miltenyi Biotech.

2.2.3 Virological methods

2.2.3.1 Egg inoculation for virus cultivation

Egg inoculation was applied in order to grow virus stocks. Viruses can only replicate in living cells. The cells and extraembryonic membranes of the chicken embryo provide varied substrates that allow the growth of many viruses. Because of the ability to alter their tropism and to adapt to a new host species, many viruses become capable of growing in chicken embryo tissues and may even attain a higher concentration than in the tissues of the natural host.

For this study all rNDVs were grown in 10-day-old embryonated chicken eggs. Hereby eggs were inoculated with 100 µl of a virus dilution (1:1000 to 1:10,000) and incubated at 38ºC. After 3 days of incubation the allantoic fluid (containing the virus) was harvested and titrated on DF1 cells by plaque assay and immunostaining against NDV NP protein (plaque assay: 2.2.3.3).

Viruses were stored at -80ºC.

2.2.3.2 Infection of cells

(Buffers and solutions: 2.1.11.4 and 2.1.11.5)

In this study A549, CEF/DF1 and Vero cells were infected with rNDVs in order to determine viral growth curves. Immunofluorescence was performed in infected A549. Moreover samples of infected A549 cells were used for qRT-PCR and Microarray studies.

Infection of adherent cells:

- 24h prior to infection $0.25x10^6$ A549/ Vero/CEF/DF1 cells were seeded into each well of a 6 well plate in order to achieve a cell monolayer (medium for A549 and Vero cells: 1XDMEM with 5ml Pen-Strep and 10% FBS, medium for CEF/DF1 cells: 1XMEM with 5ml Pen-Strep and 10% FBS); total volume per well: 2ml
- 24h later: Mock-infection and infection of A549/ Vero/ CEF cells with various rNDVs at various MOIs (for growth curves: MOI 0.01; for immunofluorescence: MOI 0.5; for qRT-PCR and Microarray: MOI 2.0). Viruses were diluted in 1XPBS/ Pen-Strep/BA for infection; inoculation volume: 200ul
- Incubation for 1h at 37ºC, rock dish every 10 min
- Remove virus and wash cells 1x with 1XPBS
- Add postinfection medium: for A549 or Vero cells 1XDMEM containing 1% FBS and 10% AF (Allantoic Fluid), for CEF/DF1 cells 1XMEM containing 1% FBS and 10% AF; total volume per well: 2ml

- Take supernatant samples (100-200ul) after different times post infection or continue with fixing cells for immunofluorescence; cell fraction was mixed with 2x protein loading buffer for Western Blot analysis
- Supernatant samples were stored at -80°C

Infection of human dendritic cells in suspension:

- After 5 to 6 days in culture, cells were infected with rNDVs at a multiplicity of infection (MOI) of 2 for 45 min in serum-free RPMI (0.5 x 10^6 cells per sample) in eppendorf tubes
- Then procedure as described above for adherent cells

2.2.3.3 Titration of infectious supernatants

(Buffers and solutions: 2.1.11.4 and 2.1.11.5)

All rNDVs were grown in 10-day-old embryonated chicken eggs and titrated on CEF/DF1 cells by plaque assay and immunostaining against NDV NP protein. The same occurred for titration of supernatants from infected cells. Plaque assay overlay medium based on Dulbecco's modified Eagle medium MEM F12 was supplemented with Mg_2SO_4 (final concentration 0.03M), Trypsin (final concentration 2.5µg/ml) and dextran (final concentration 0.02%) in order to support plaque formation of rNDV (Kournikakis & Fildes, 1988).

Set-up:

Dilution of virus inoculum

- Take a 24 well dish, and add 450 µl of PBS/BA/P-S to each well. Orient dish to put samples across the top of the dish (max 4 samples per dish), giving 6 wells for dilution of each sample
- Add 50 µl of each virus to be assayed to one well in the top row of wells, giving a 10 -1 dilution. Add virus to each sample, then swish the dish to mix. Take 50 µl aliquot from these wells into the next well down, giving a 10 – 2 dilution. Swish dishes again, and carry on down the plate diluting to 10-6.

Inoculation of cells

- Wash cells with 2 ml PBS
- Add 200 µl of each virus dilution to each well with DF1/CEF cells (48h prior to infection $0.25x10^6$ cells were seeded into each well) in a 6-well dish. Rock for 1h at 37°C, rocking at least every 10 minutes.
- Remove inoculum
- Wash again in 2 ml PBS

Addition of overlay

- Add overlay medium to cells (cooled down to a temperature of 37ºC after adding the oxoid agar; agar had to be boiled in order to be fluid)
- Incubate plates at 37°C for 3 days or until plaque formation

Fixing of plaques and immunostaining

- Fix cells with 4% formaldehyde solution in 1XPBS. Add 2 ml of solution to each 35 mm well while overlay is still in place. Incubate at RT for 3h – no need to rock dishes
- Remove overlay
- Wash 1x with 1 ml 1X PBS.
- (If the target protein is intracellular, add a permeabilisation step: add 2 ml of 0.5% Triton-X in 1XPBS to wells, leave it for 3-5 min at RT
- After permeabilization wash 3x with 1 ml 1XPBS
- Block with 5 % skimmed milk in 1XPBS / 0.05 % Tween 20 (2 ml per 35 mm dish for 1 h at RT). No rocking necessary
- Wash 1x with 1 ml 1XPBS.
- Incubate overnight at 4 $^{\circ}$C with dilution of primary antibody in 5 % skimmed milk in 1XPBS / 0.05 % Tween 20 solution
- Wash 1x with 1 ml 1XPBS.
- Incubate with HRP-conjugated secondary antibody in 5 % skimmed milk in 1XPBS / 0.05 % Tween 20 solution, 1 h RT. No rocking required.
- Wash 3x with 1 ml 1XPBS.
- Add 500 µl of TRUE BLUE (KPL) stain.
- Incubate 1-10 minutes RT (or as soon as stained plaques are visible)
- Remove stain
- Quickly wash in H_2O, aspirate H_2O off pronto.

- Air dry, inverted. (The stain is apparently sensitive to water, and prolonged contact bleaches the colour out).

3. Results

3.1 The effect of Nipah virus V and W proteins on the human host immune response

3.1.1 Characterization of recombinant Newcastle Disease Virus expressing Nipah virus V and W proteins

The work with Nipah virus itself proves to be difficult since the virus is a classified BSL-4 agent. To examine the function of Nipah virus proteins in the context of a virus infection under BSL-2 conditions, rNDVs producing Nipah V or W proteins, have been developed using the previously described NDV system (Park *et al.*, 2003a; Park *et al.*, 2003b). Infectious cDNAs for NDV have recently been developed (Krishnamurthy *et al.*, 2000; Nakaya *et al.*, 2001; Peeters *et al.*, 1999; Romer-Oberdorfer *et al.*, 1999) and allow the introduction of foreign genes into the NDV genome (Krishnamurthy *et al.*, 2000; Nakaya *et al.*, 2001; Schickli *et al.*, 2001). Here recombinant NDVs expressing NiV V and W proteins were constructed and characterized. The results will demonstrate that NiV V and W proteins contribute to a better viral growth during infection in human cells.

The open reading frames (ORFs) of NiV V and W were introduced into an editing deficient NDV that cannot produce the V transcript (Fig. 3). More specifically these ORFs were inserted between the P and M gene. To make sure that solely the NiV V or NiV W transcript was generated, both the editing site and NiV C ORF were mutated. As a control virus served an editing deficient NDV in which the NDV V ORF was reinserted between the HN and L gene. Despite the different insert location this virus behaved like

wildtype virus. This justified its use for the following experiments. Recombinant NDVs expressing NiV V or W proteins were compared to the rNDV expressing the NDV V protein.

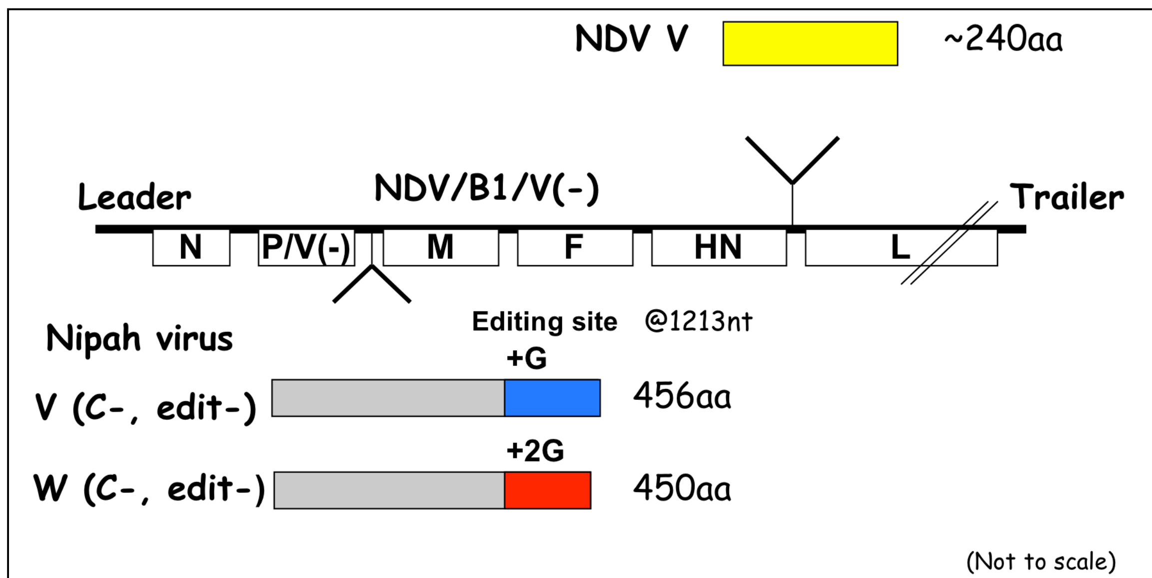


Fig. 3: Schematic representation of recombinant NDVs expressing NDV V, NiV V or NiV W proteins.
As backbone virus served NDV/B1/(V-). This virus cannot produce the V transcript since its editing site was mutated by changing aa A to G. NDV V ORF was inserted between the HN and L gene. NiV V and NiV W ORFs (with mutated C ORF and editing site) were inserted between the P and M gene, respectively. For details, see Materials and Methods. NiV V and W share the same N terminal region (shown in grey) and have a unique C terminus (shown in blue or red). For future presentation NDV V is colored in yellow, NiV V in blue and NiV W in red.

For titration of these rNDVs a specific NDV plaque assay was developed in immortalized chicken embryo fibroblast (CEF) cell line, based on a previously published report (Kournikakis & Fildes, 1988) (Fig. 4).

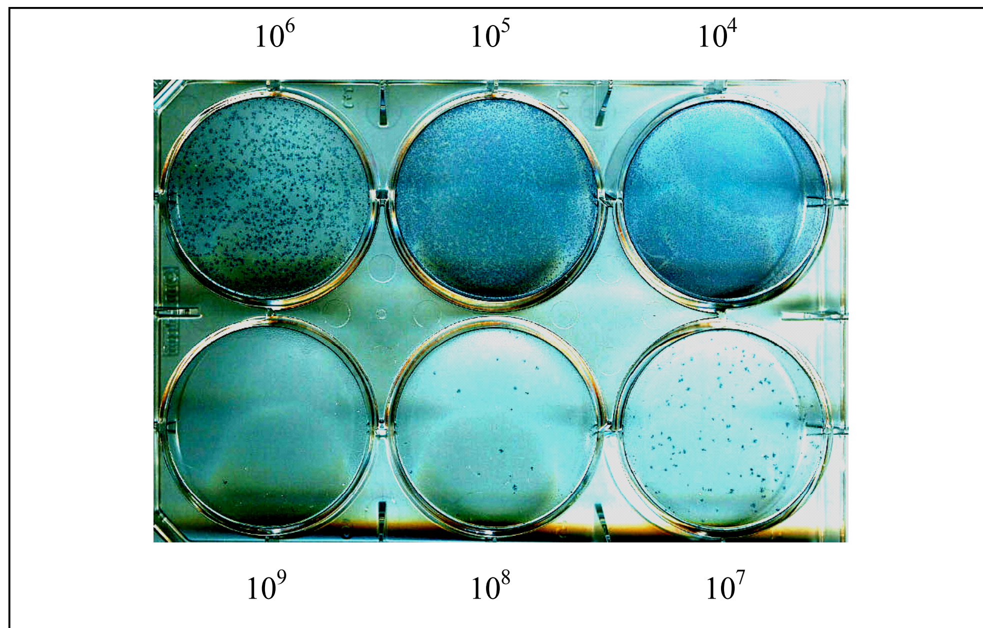
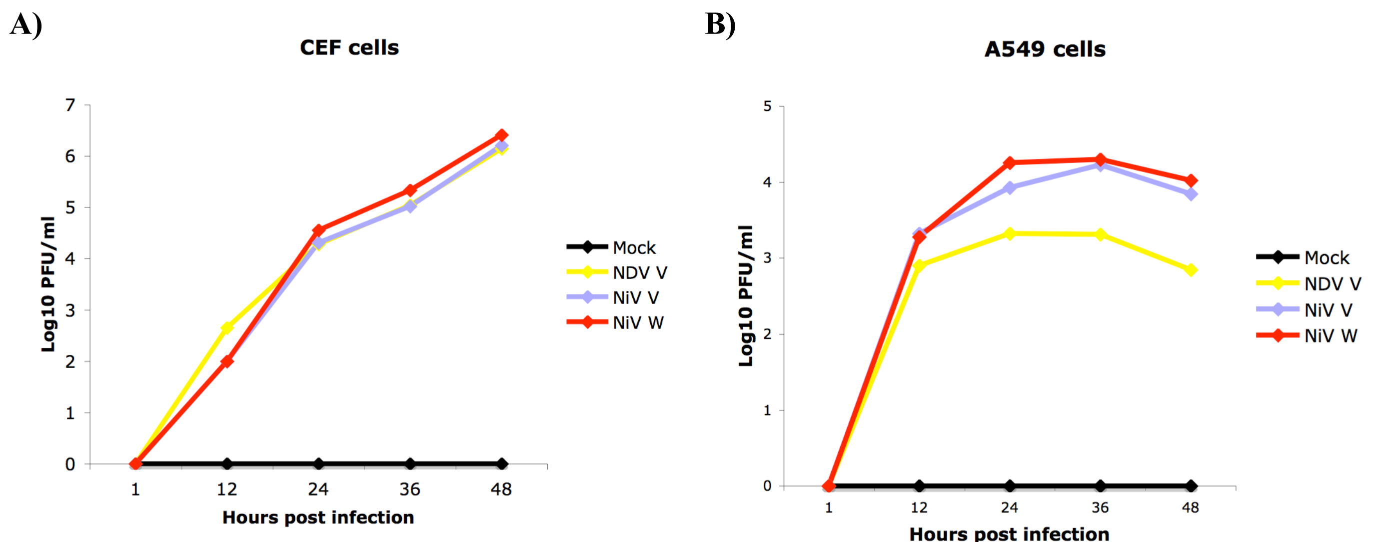


Fig. 4: Plaque assay and immunostaining for rNDVs.
The image shown above represents an example for the immunostaining of plaques formed by rNDV/NiV W. Plated CEF cells were infected with allantoic fluid of virus-inoculated 10-day old chicken eggs in 10-fold dilutions. After 72 hours of incubation cells were fixed, permeabilized and stained using an antibody against NDV NP protein.

The growth characteristics of these rNDVs were determined in three different cell lines, which were infected with rNDV/NDV V, rNDV/NiV V and rNDV/NiV W at a low MOI of 0.01 (Fig. 5). The cell supernatants were titrated after certain time points. It can be seen that all rNDVs have approximate similar growth kinetics in CEF cells with a maximum titer of about 10^7 PFU/ml at 48 hours post infection (hpi) (Fig. 5 A). This was to be expected because avian viruses were used. Human A549 cells display an interesting phenomenon (Fig. 5 B). As mentioned before wildtype NDV has a species-specificity for

avian cells and normally does not grow well in human cells (Park *et al.*, 2003a). Interestingly rNDVs expressing NiV V or NiV W grow to significantly higher titers than rNDV expressing NDV V. Around 36 hpi the maximum titer for rNDVs expressing NiV V or NiV W lies between 10^4 and 10^5 PFU/ml compared to approximate 10^3 PFU/ml for rNDV expressing NDV V. This difference of 1½ log in titer suggests that NiV V and NiV W proteins render the virus into a better growing virus in human cells. This potentially could also explain the high lethality of Nipah virus in humans. In contrast, like in CEF cells all rNDVs grew to similar (but generally lower) titers in Vero cells (Fig. 5 C). Vero cells cannot produce interferons, so it was to be expected that the rNDVs, whether or not they encode for IFN antagonists, were able to grow to similar titers.



C)

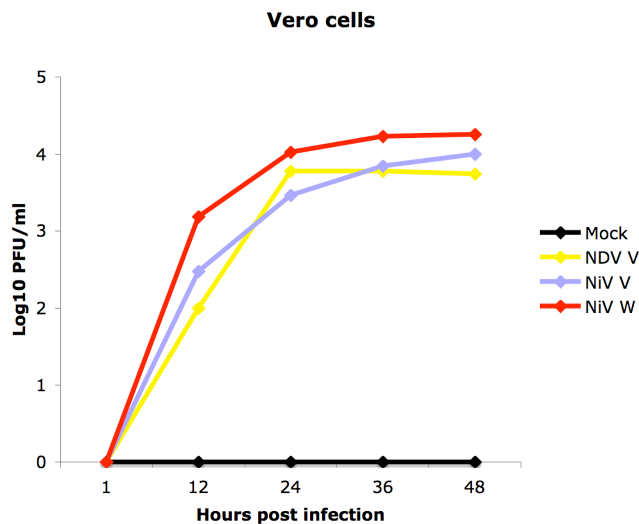


Fig. 5: Growth characteristics for rNDVs in different cell lines.
CEF, A549 or Vero cells were infected with rNDV/NDV V, rNDV/NiV V or rNDV/NiV W at an MOI of 0.01 (A, B and C). At different time points after infection, cell supernatants were collected and viral titers were determined by NDV plaque assay and immunostaining using an antibody against NDV NP protein. The growth curves represent one of two individually performed experiments.

Given the growth curve results for rNDVs expressing NiV proteins in A549 cells, it was of specific interest to analyze the impact of these proteins in a human host. Therefore all following experiments were performed in human A549 cells.

A549 cells were infected with the rNDV constructs (MOI 0.5) and stained with specific antibodies against NiV V and NiV W protein and counterstained against NDV HN protein (Fig. 6). The HN protein can be found in the cytoplasm of all infected cells (Fig. 6 A, B, C). Similarly, the NiV V protein of rNDV/NiV can predominantly be seen in the cytoplasm (Fig. 6 B). In contrast, NiV W localizes exclusively in the nucleus (Fig. 6 C).

This observation correlates with published data about the localization of NiV V and NiV W proteins in plasmid-transfected cells (Shaw *et al.*, 2005).

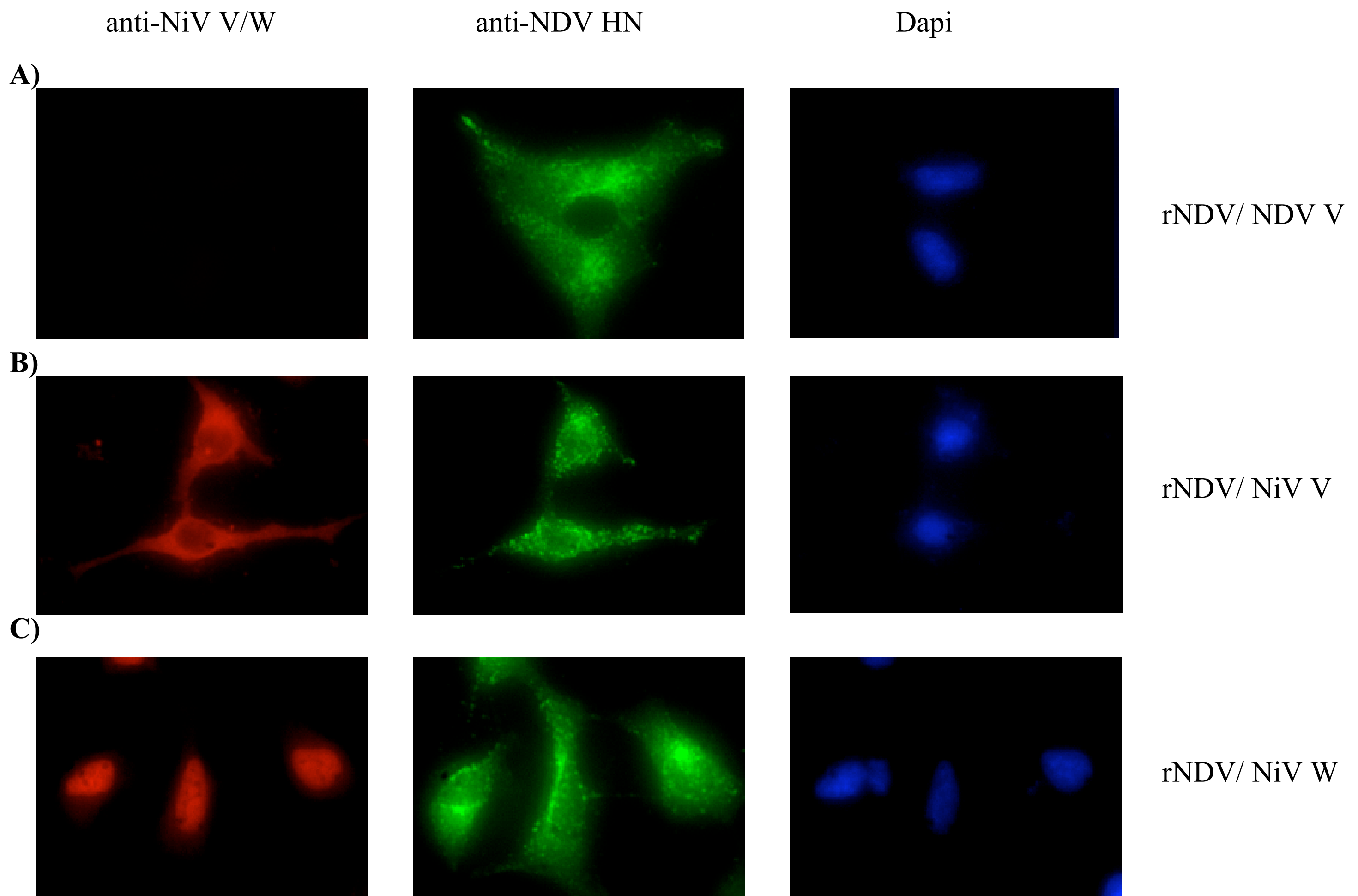


Fig. 6: Localization of rNDV proteins in A549 cells.
A549 cells were infected with rNDV/NDV V (A), rNDV/NiV V (B) or rNDV/NiV W (C) at an MOI of 0.5. Cells were fixed and permeabilized after 22 hpi and stained using antibodies against NDV HN, NiV V and NiV W protein.

3.1.2 Nipah V and W proteins potently downregulate the interferon system in human A549 cells

The innate immune system plays critical roles in detecting viral infections and evoking anti-viral responses and the host IFN system serves as one of the primary defense mechanisms (Basler & Garcia-Sastre, 2002). However, viruses have developed several strategies in order to circumvent the IFN-induced antiviral state (Garcia-Sastre, 2001; Katze *et al.*, 2002; Levy & Garcia-Sastre, 2001). Among negative-stranded RNA viruses, several IFN antagonists have been identified, such as the influenza virus NS1 protein (Garcia-Sastre *et al.*, 1998), the Bunyamwera virus NS proteins (Weber *et al.*, 2002) or the Ebola virus VP35 protein (Basler *et al.*, 2000). Moreover, there are numerous IFN antagonists within the paramyxoviruses (Gotoh *et al.*, 2002). The paramyxovirus IFN antagonist functions are all encoded by the P gene and are most predominantly carried out by one of the accessory V and C proteins. However, the P gene of Nipah virus is predicted to encode four proteins, namely P, V, W and C (Harcourt *et al.*, 2000; Wang *et al.*, 2001). All four proteins have been demonstrated to have IFN antagonist activity (Park *et al.*, 2003b; Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). For this chapter it was of interest to examine the effect of NiV V and W proteins when they are expressed from recombinant Newcastle disease virus and it will be shown that NiV V and especially NiV W exert a potent inhibition on the host immune response.

The effect of NiV V and NiV W proteins on the immune response was examined in human A549 cells. The cells were infected with the respective viruses at a high MOI (Table 1). Immunofluorescence experiments confirmed that 100% of cells were actually infected (data not shown).

Infection with:	**Label**
Mock	Mock
rNDV/NDV	NDV V
rNDV/NiV V	NiV V
rNDV/NiV W	NiV W

Table 1: Infection of A549 cells or human dendritic cells.
Cells were mock infected or infected with the above listed rNDVs at an MOI of 2. For purpose of presentation the different rNDVs are colored in yellow (NDV V), blue (NiV V) and red (NiV W).

In order to study the effect of NiV V and W proteins on the transcription of host immune genes, mock infected and virus-infected A549 samples were analyzed by quantitative RT-PCR using specific primers as mentioned below (Fig. 7). Viral replication of the respective rNDVs was confirmed by using primers for viral NDV NP (Fig. 7 A). The mRNA expression levels of viral NDV NP increased for all viruses over the time course of 24 hours. NP mRNA levels were a little less for rNDV expressing NDV V compared to rNDVs expressing NiV V or W. The same observation was made for NP protein levels (Fig, 9, middle panel). This result was reproducible and in agreement with the growth curve data where rNDV/NDV V grew to lower titers compared to rNDV/NiV V or W (Fig. 5). Most importantly mRNA expression levels of IFN-alpha and IFN-beta were

strongly reduced in the presence of NiV V and W over the time course (Fig. 7 B and C). Both NiV proteins also have been seen to downregulate the transcription of essential transcriptional activators such as STAT1 (Fig. 7 D), IRF1 (Fig. 7 E), IRF7 (Fig. 7 F) and NFkappaB1 (also known as p50, Fig. 7 G). Furthermore, mRNA expression levels of interferon-stimulated genes were low in the presence of NiV V and W. These included ISG54, ISG56, RANTES (also known as CCL5), RIG-I, MxA, TNF-alpha, IL-6 and CCL3 (also known as MIP1-alpha) whereas the relative copy numbers for last three genes were lower compared to the others (Fig. 7 H-O). For some genes (RANTES, ISG54 and IL-6), the transcription levels seemed to decrease between 20 and 24 hpi. This is possibly due to cell apoptosis and may not reflect a significant downregulation. Interestingly, in all cases NiV W seemed to be a stronger inhibitor of gene transcription than NiV V. In contrast, NDV V protein induced an immune response by upregulating the transcription of all these genes.

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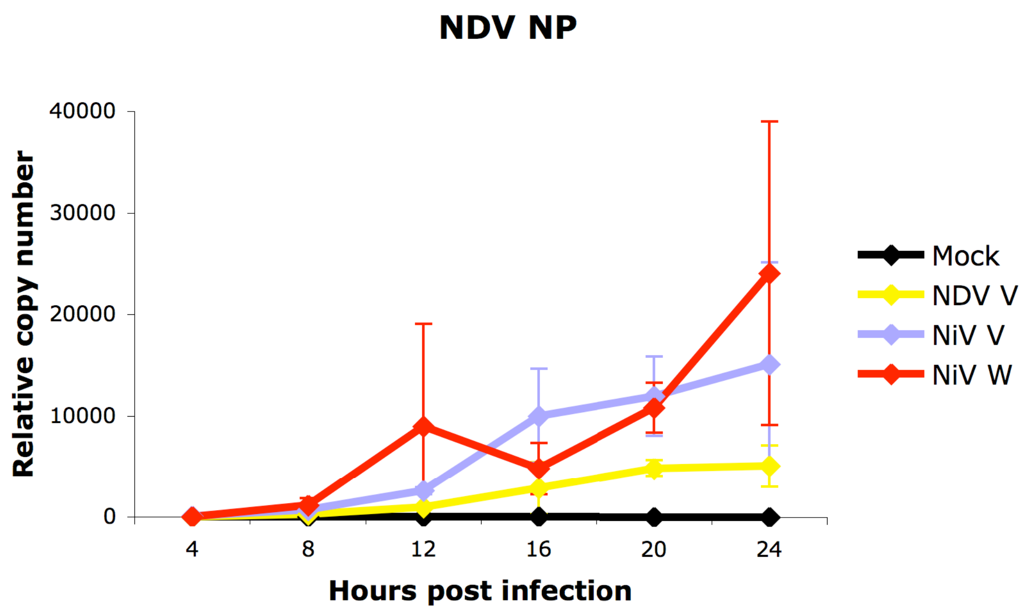

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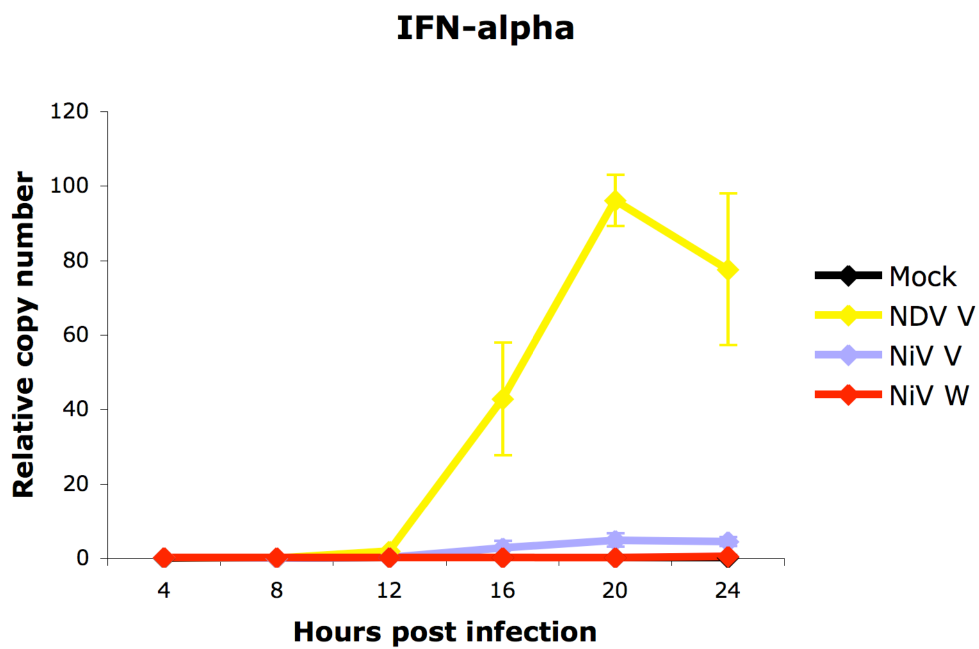

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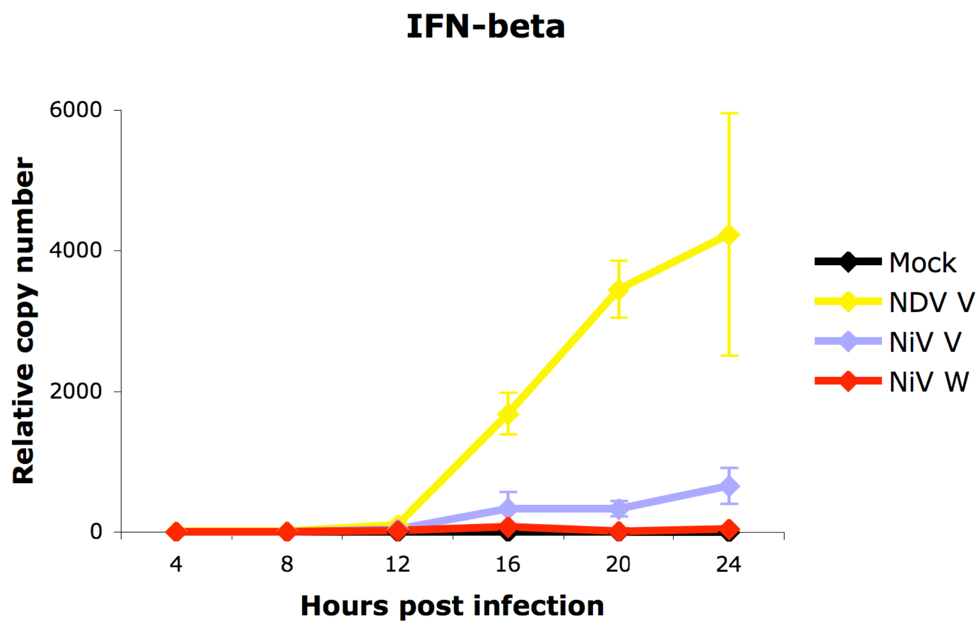

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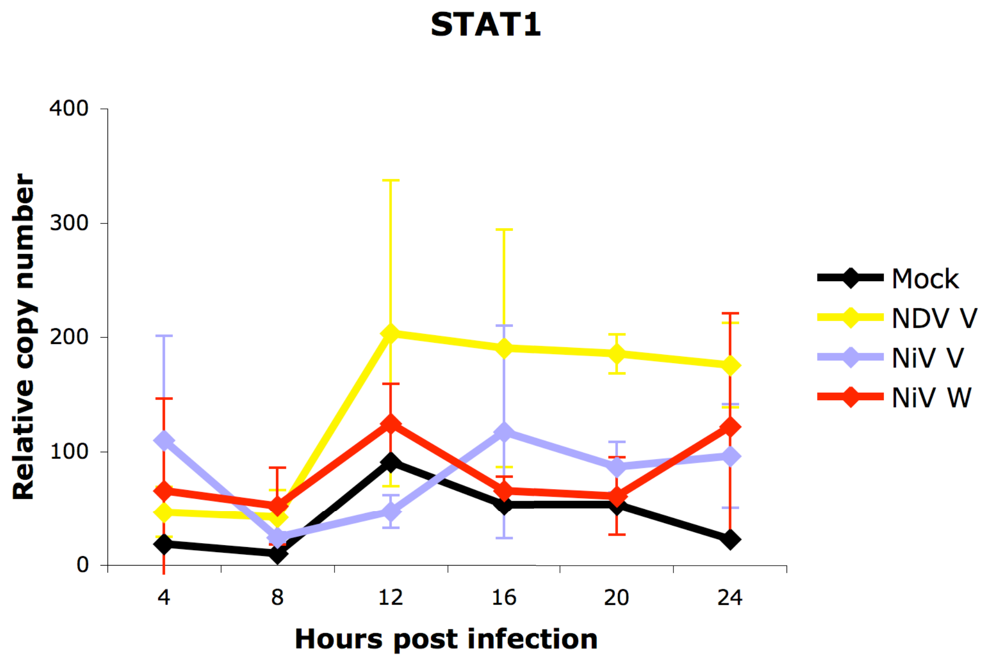

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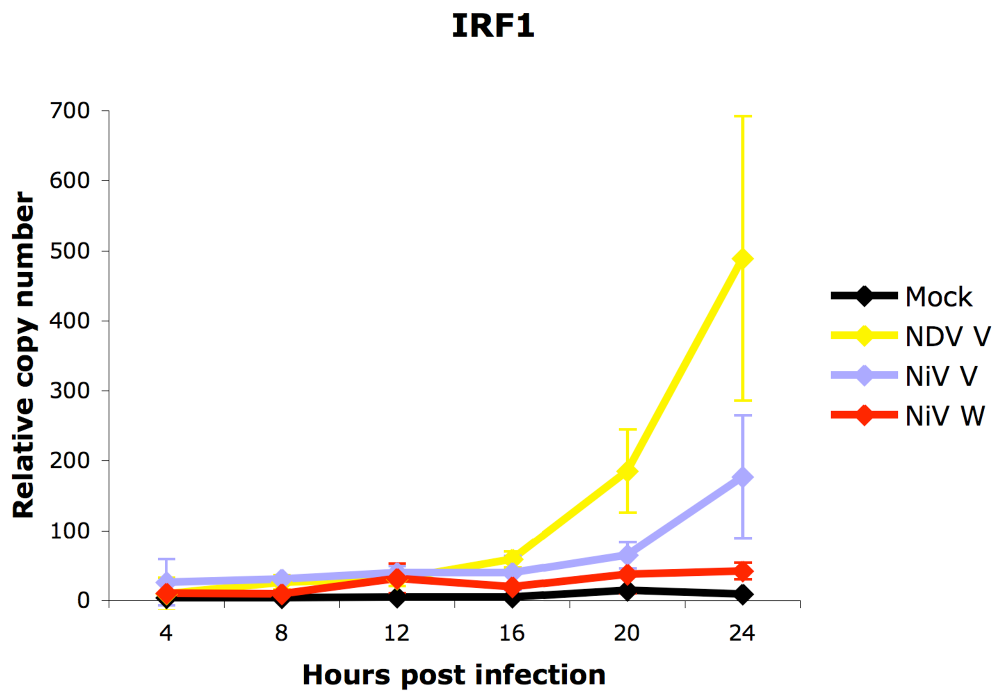

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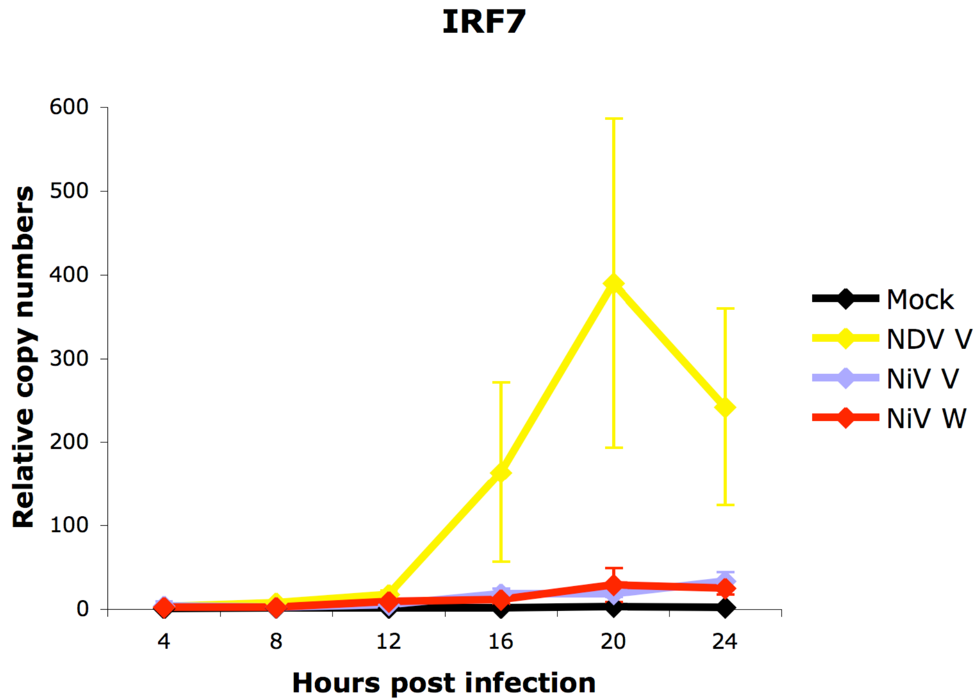

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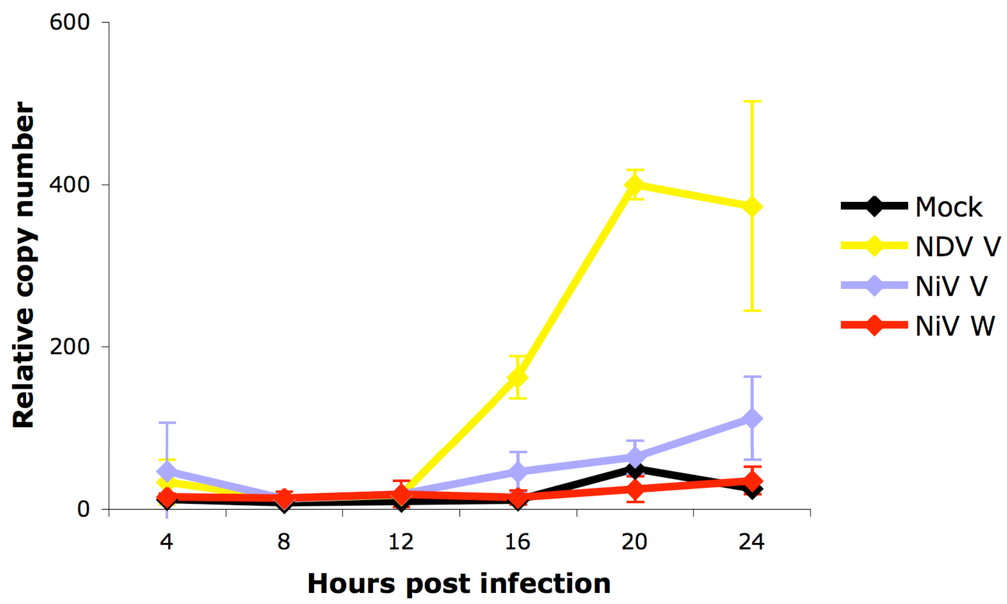

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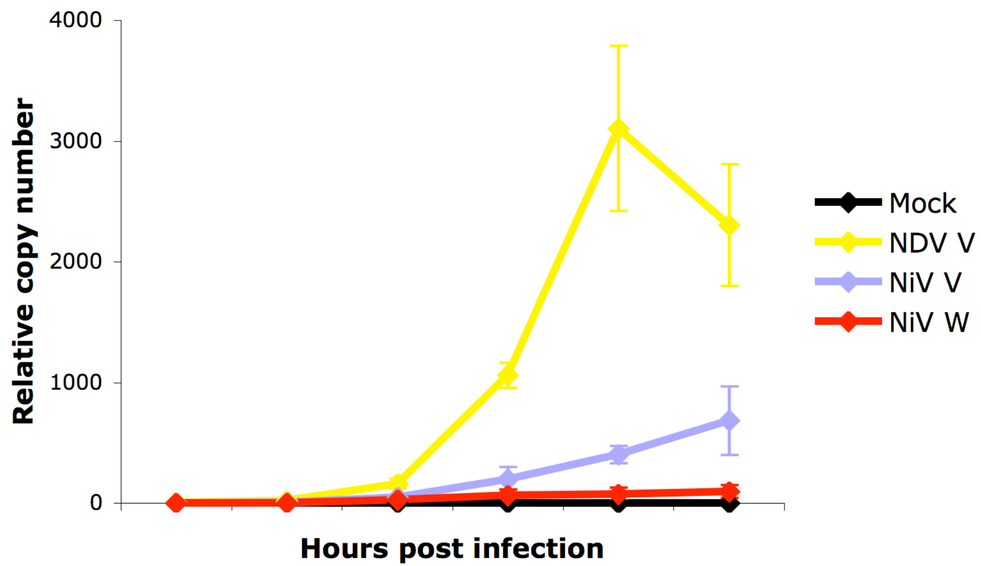

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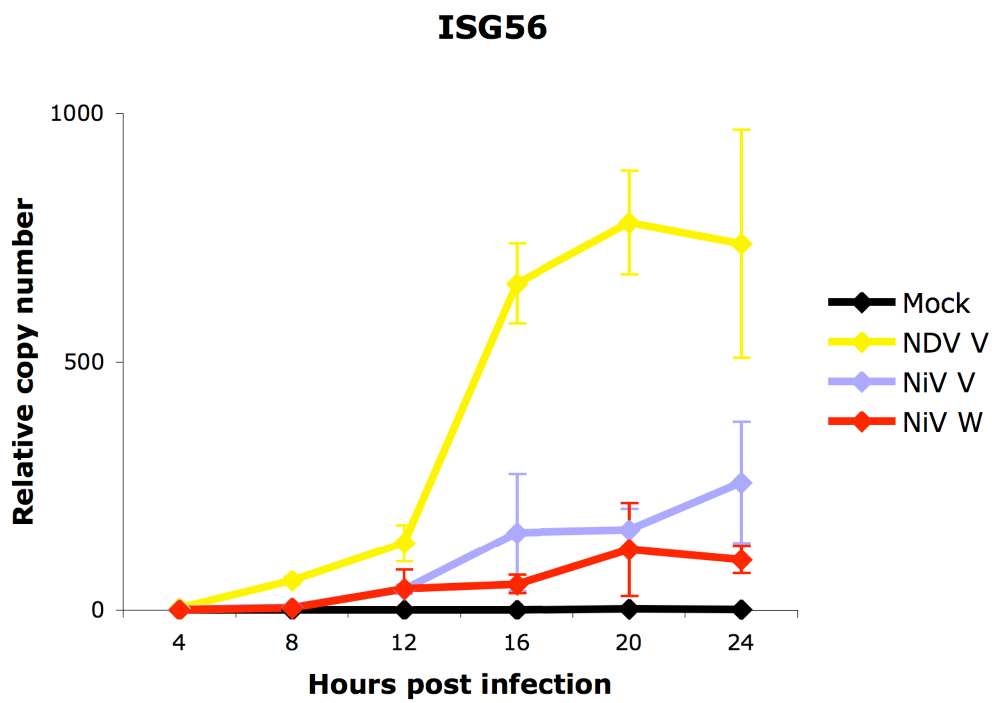

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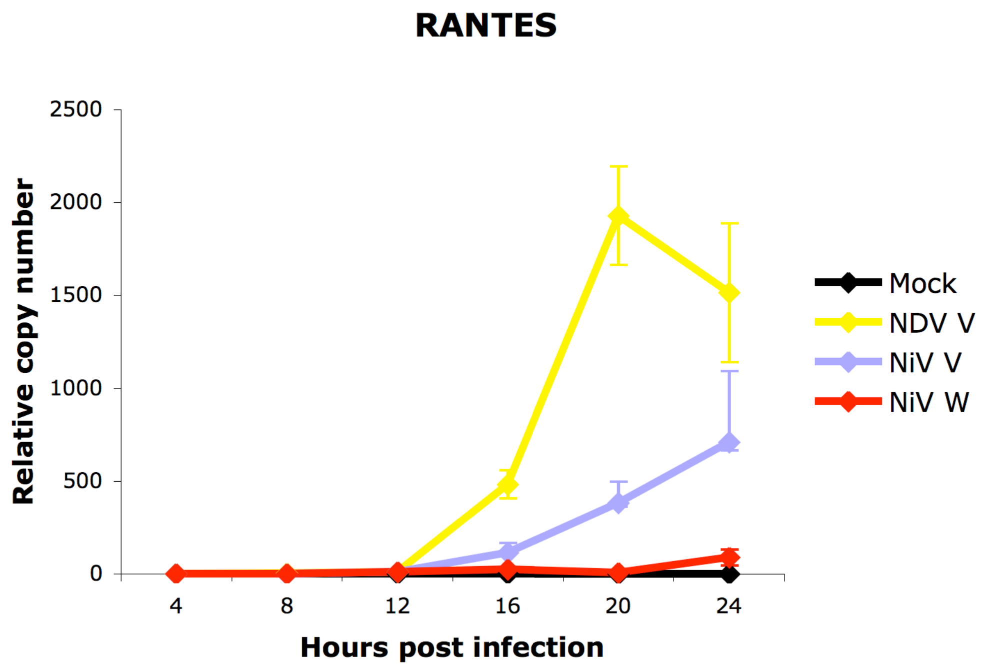

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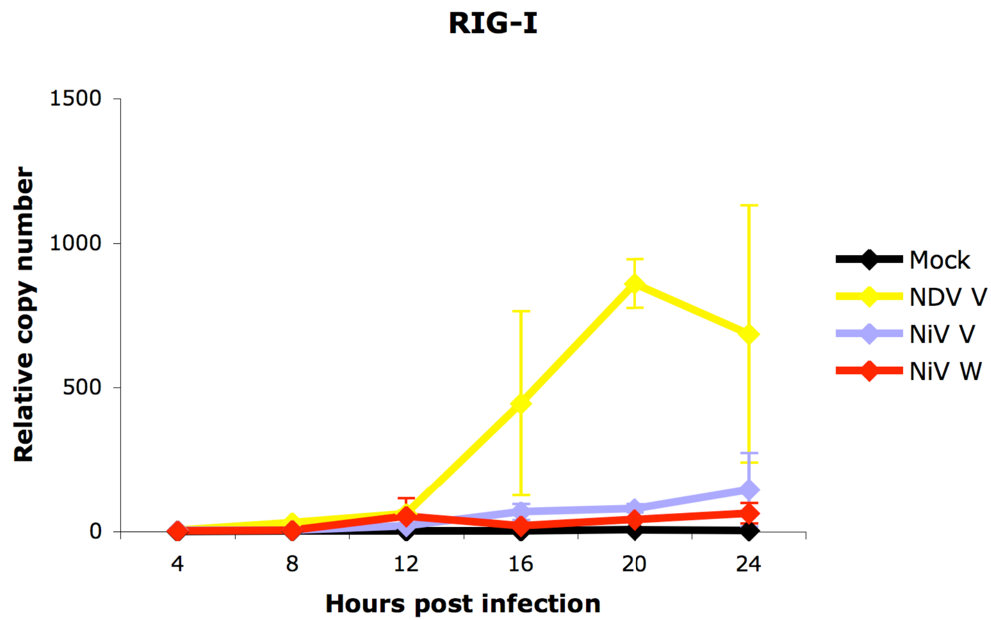

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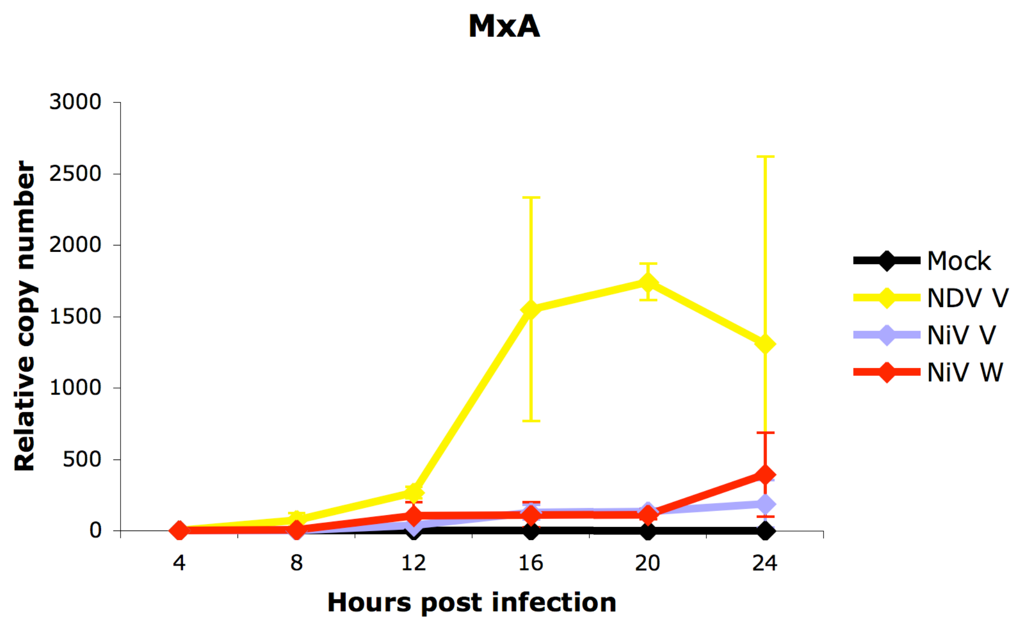

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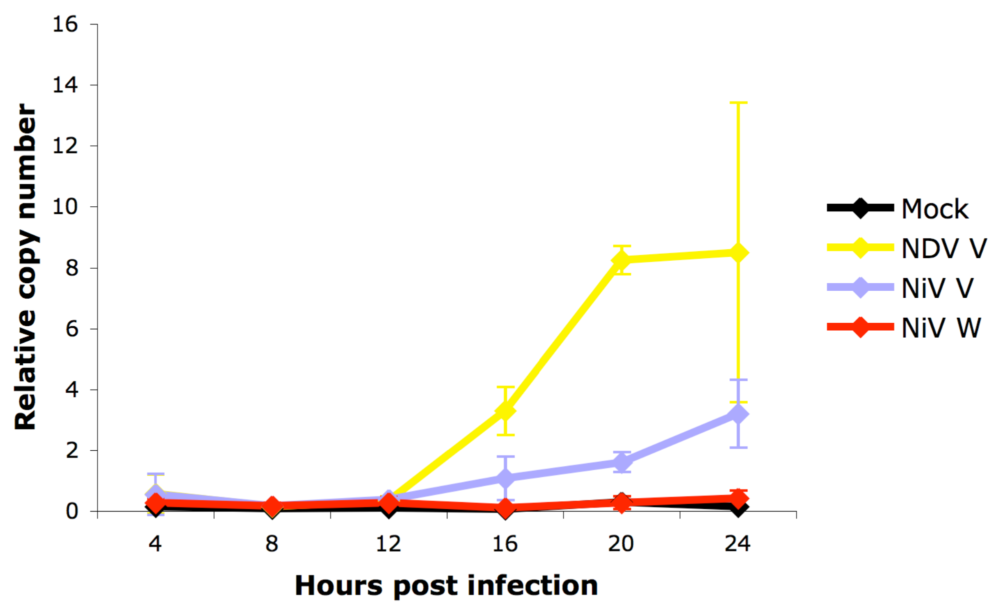

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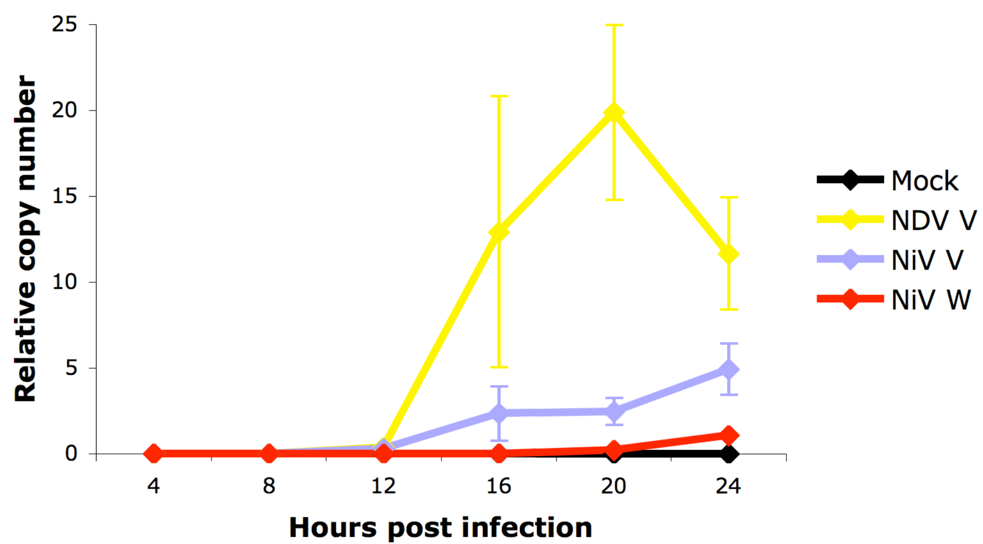

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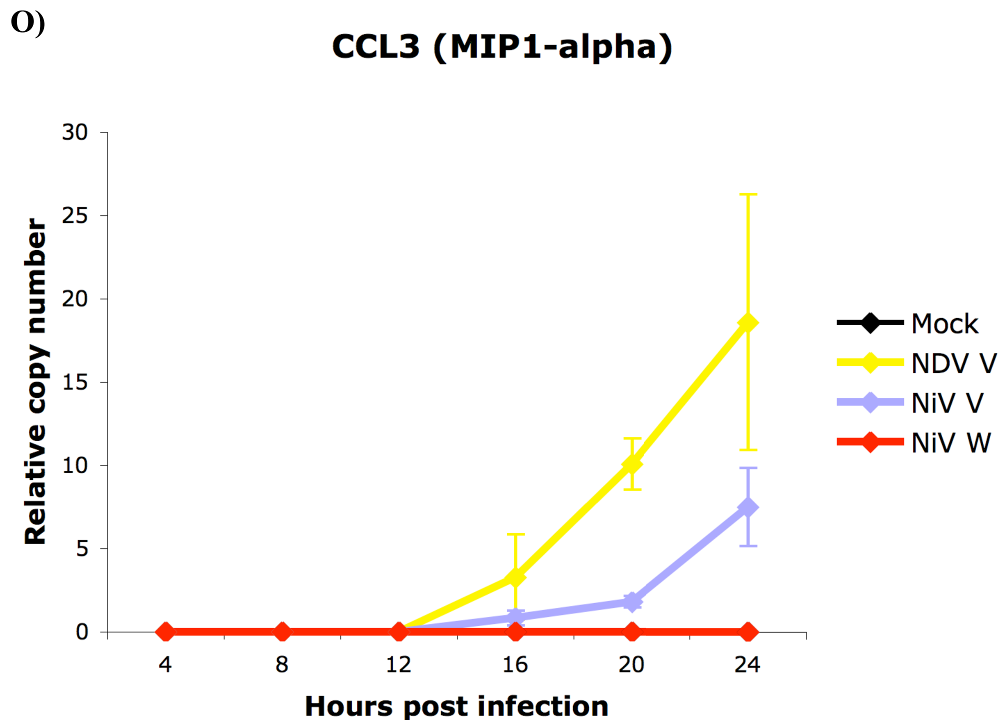

Fig. 7: NiV V and W proteins downregulate the transcription of important immune genes.
A549 cells were either mock infected or infected with rNDV/NDV V, rNDV/NiV V or rNDV/NiV W at an MOI of 2. Infection was carried out in triplicates. At different time points cell lysates were collected. RNAs of each triplicate were isolated and used to generate cDNAs to specifically test for gene expression of selected immune genes by qRT-PCR (A-O). Values indicate changes in gene expression in mock infected and virus-infected A549 cells compared to expression of three housekeeping genes (actin, tubulin, rsp11). Error bars represent standard deviations for triplicate samples.

In order to analyze the up- and downregulation on a bigger scale, a specific human immune Microarray was performed (Fig. 8 and Table 2). Samples of rNDV-infected A549 cells were compared and normalized to mock-infected A549 cells. Figure 8 gives a general trend for the up- or downregulation of all 1,070 genes being tested. Each spot represents a certain gene within the array and the diagonal represents the baseline gene expression determined by mock-infected samples. Everything above the baseline

accounts for a gene upregulation whereas everything below the baseline accounts for a downregulation. The graph shows that the infection with rNDV/NDV V caused a greater upregulation of genes compared to infections with rNDV/NiV V or W (Fig. 8 A-C). In fact, the presence of NiV W protein seemed to inhibit the expression of the tested immune genes to a greater extent than NiV V.

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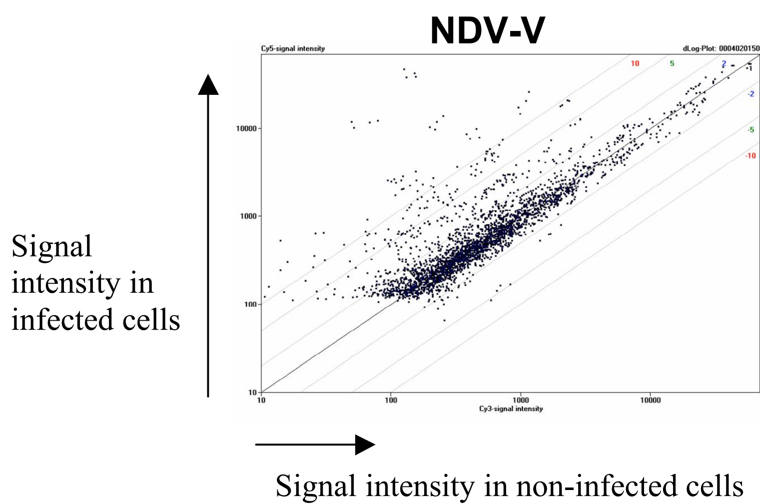

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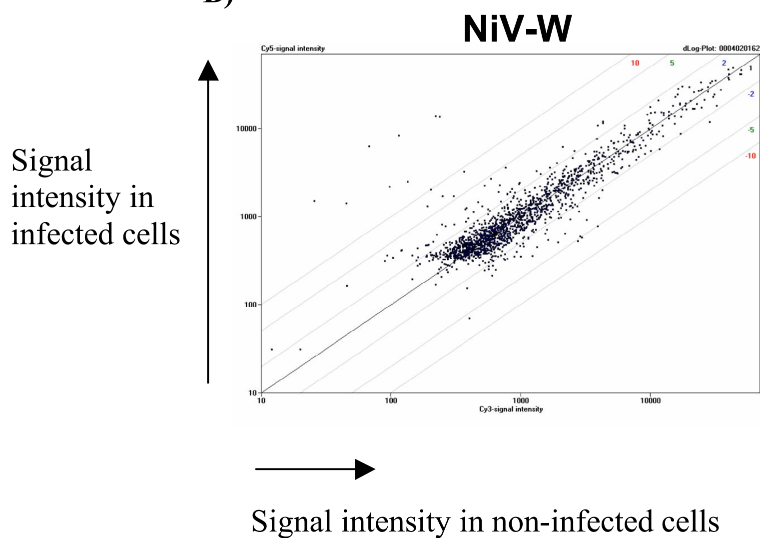

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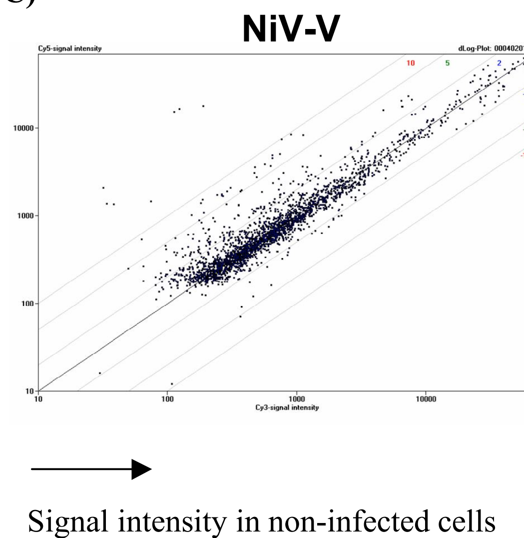

Fig. 8: Global inhibitory effect of NiV V and W proteins on host immune gene expression.
A549 cells were infected with rNDV/NDV V, rNDV/NiV V or rNDV/NiV W at an MOI of 2. Cells were harvested 18 hpi and analyzed by Microarray analysis against uninfected A549 cells, using an immunology Microarray panel of 1,070 genes (Miltenyi Biotec). *y* axes show signal intensities in infected cells, and *x* axes show signal intensities in uninfected cells.

1,070 genes were tested in the Microarray, but the specific interest was in genes that were significantly downregulated by NiV V or W compared to NDV V (Table 2). For this selection genes that were ≥ 1.7 fold upregulated in their expression by NDV V served as starting point. A 1.7 fold upregulation of gene expression compared to baseline gene expression was considered to be significant. These values were set as 100% and compared to values obtained for NiV V and W. Table 2 enlists genes that were significantly downregulated in their expression by both NiV proteins compared to NDV V. Hereby, a significant downregulation was considered to be ≥ 50%. In this way the number of genes of interest was narrowed down to 38. Both NiV V and W proteins were able to significantly reduce the transcription of various genes compared to NDV V protein. Noticeably, genes that were previously analyzed by qRT-PCR were also part of this selection (Table 2, green shaded boxes). It should be mentioned for the Microarray that due to quality issues no gene expression data was obtained for IFN-alpha and TNF-alpha. The genes for ISG54, RIG-I and MxA were also not included in the Microarray. IL-6 is not shown in Table 2 because the array did not show a significant downregulation in the presence of NiV V or W. Quantitative RT-PCR also showed low relative copy numbers for TNF-alpha gene transcription, which might imply that the downregulation was not significant (Fig. 7 N). However, downregulation of transcription of the remaining

eight genes (NFkappaB1, STAT1, CCL3, RANTES, IRF1, IFN-beta, IRF7 and ISG56) by NiV V and W protein was seen for both qRT-PCR and Microarray. NiV V and W proteins reduced the expression of numerous other genes (Table 2). Their gene products are involved in various parts of the host immune response such as cytokine/chemokine signaling (e.g. CCL2), apoptosis (e.g. CASP4) or activation of transcription (e.g. ATF3). Within this selection shown in Table 2 the transcription of some genes was more potently downregulated by NiV W than NiV V (Table 2, genes shown in red). Hereby, a ≥ 1.7 fold difference between NiV V and W values was considered to be significant. Interestingly, besides IFN-beta and chemokines NiV W protein is specifically involved in the downregulation of important transcriptional factors such as ATF3, JUNB or members of the NFkappaB family.

IAP1	**CCL5 (RANTES)**
GADD34	CXCL12
CASP4	IRF1
MCL1	TAP1
PLAUR	SOD2
CD38	C1S
PAI1	B2M
ATF3	**IFNB1**
JUNB	CEACAM1-CEACAM2
FIP2 (NEMO2)	GN6ST
IKAPPA B-ALPHA	IRF7
NFKAPPA B1 (p50)	PDCD1LG2 (B7DC)
NFKAPPA B2	PARG1
NFKAPPA B3 (REL A)	MAP3K8
SOCS1	IGFBP6
STAT1	**ISG56 (IFIT1)**
CCL2	PIAS1
CCL20	IFITM1
CCL3 (MIP1-ALPHA)	IFITM2-IFITM3

Table 2: Immune gene Microarray: Genes significantly downregulated by NiV V and W compared to NDV V.
Microarray data (see Fig. 9) for 1,070 genes was narrowed further down. Significantly upregulated genes (compared to mock) were selected. Hereby, a 1.7 fold upregulation of gene expression was considered to be significant. Then values of significantly upregulated genes in the presence of NDV V were set as 100% and compared to values obtained for NiV V and NiV W. Hereby, a downregulation of gene expression of ≥ 50% by NiV V or W proteins (compared to NDV V) was considered as significant. For comparison between NiV V and W a 1.7 fold difference of gene expression was considered as significant. Green shaded box = genes that were also tested by RT-PCR, red letters = genes that were more potently downregulated by NiV W compared to NiV V

The effect of NiV V and W proteins on the host immune response was also analyzed at the level of translation (Fig. 9). Hereby, the focus was on RIG-I and MxA, two representatives of IFN-stimulated genes. As previously mentioned, protein analysis confirmed that all viruses expressed viral NDV NP protein and that the levels increased over the time course of 24 hours (Fig. 9, middle panel). NDV NP levels for rNDV/NDV V were a little lower compared to rNDV/NiV V or NiV W. This result matched the data obtained by qRT-PCR (Fig. 9 A).

Protein expression of RIG-I, one of the major proteins involved in virus recognition (Kumagai *et al.*, 2008), was potently downregulated by NiV V and NiV W proteins (Fig. 9, top panel). In contrast, NDV V protein induced RIG-I expression in an increasing manner over time. RIG-I seemed to be induced at later time points in the presence of both NiV proteins, but still to a much lesser extent as seen for NDV V.

MxA belongs to the IFN-induced genes and has an important antiviral role for many RNA viruses (Haller *et al.*, 2007; Hoenen *et al.*, 2007; Tumpey *et al.*, 2007). MxA protein expression was significantly less in the presence of both NiV V and NiV W

protein. In contrast, NDV V protein induced increasing amounts of MxA over time (Fig. 9, bottom panel). As seen for RIG-I there was some induction of MxA protein expression at later time points. However, levels were never as high as seen in the presence of NDV V protein. Most importantly the fact that less RGI-I and MxA were detectable might be most likely due to the transcriptional block determined by qRT-PCR (Fig. 7 K and L). The production of less protein is the consequence of less mRNA synthesis.

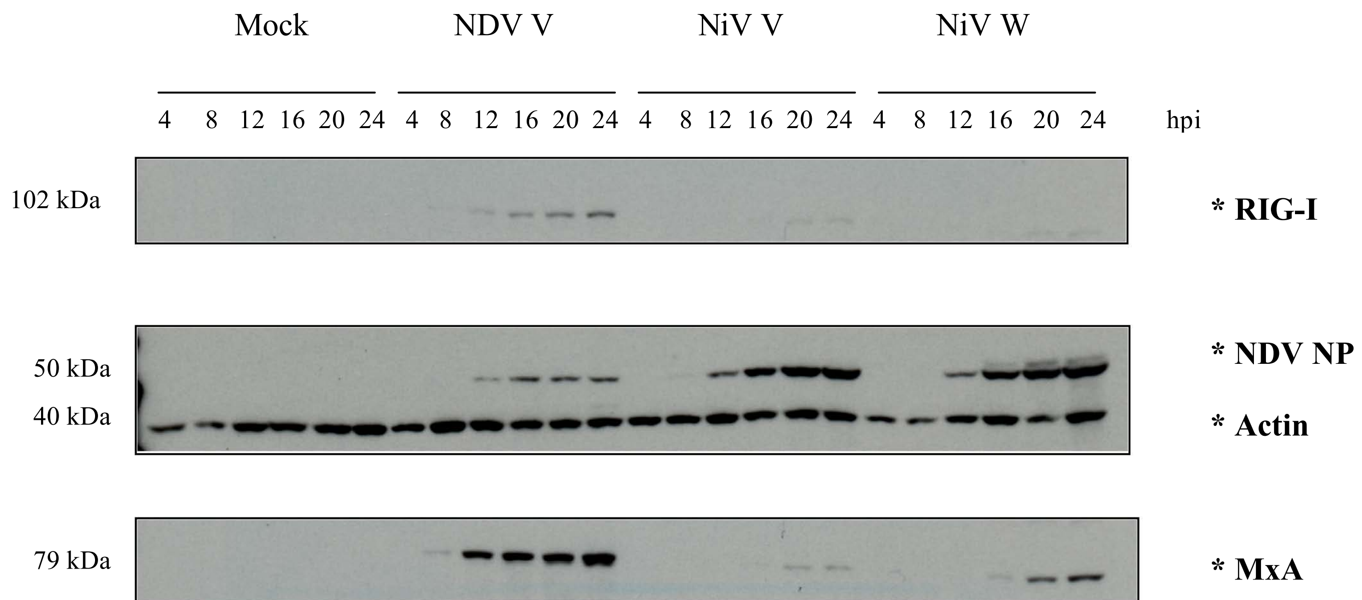

Fig. 9: NiV V and W proteins downregulate the expression of important immune proteins.
A549 cells were either mock infected or infected with rNDV/NDV V, rNDV/NiV V or rNDV/NiV W at an MOI of 2. Infection was carried out in triplicates. At different time points cell lysates for each triplicate were collected. Pooled cell lysates were subjected to Western Blot analysis using antibodies against NDV NP, RIG-I, MxA and actin protein, respectively.

3.1.3 Nipah V and W proteins potently downregulate the host immune response in human dendritic cells

Dendritic cells constitute a unique cell system because they represent a link between the innate and the adaptive immune response (Banchereau & Steinman, 1998; Palucka & Banchereau, 1999). B- and T-lymphocytes are part of the adaptive immunity, but their function is under the control of dendritic cells. In addition DCs contribute to the antiviral innate immune system by secreting IFN-alpha and IFN-beta in response to viral infection. Paradoxically, instead of inducing host resistance, DCs also provide a good environment for viruses and pathogens, in general. Cells of the DC system may be hosting latent cytomegalovirus (Soderberg-Naucler *et al.*, 1997), human immunodeficiency virus type I (Cavrois *et al.*, 2008), measles virus (de Witte *et al.*, 2008), respiratory syncytial virus (Munir, Le Nouen et al. 2008) or influenza virus A (Munir *et al.*, 2008; Wang *et al.*, 2008).

Here human dendritic cells were infected with rNDVs expressing NiV V and W proteins and it will be shown that both proteins potently abrogate the host immune response.

Considering the importance of dendritic cells it was of interest to examine the effect of NiV V and W proteins on these cells.

Similar to the experiments being performed in human A549 cells, isolated human dendritic cells (DCs) were infected with the respective rNDVs expressing NDV V, NiV V or NiV W protein (Table 1).

Quantitative RT-PCR data showed that both NiV V and NiV W proteins have an inhibitory effect on the transcription of important immune genes over time (Fig. 10). Most importantly mRNA levels of both IFN-alpha and IFN-beta were reduced in the presence of NiV V and W (Fig. 10 A and B). The reason for higher measured IFN-alpha values compared to IFN-beta might be due to the use of a pan anti-IFN alpha primer pair. This primer pair is reactive with all IFN-alphas and therefore detected all 13 IFN-alpha subtypes. This might have accounted for the increased value of relative copy numbers in comparison to IFN-beta. The transcription of selected ISGs such as RIG-I, MxA, RANTES, TNF-alpha, IL12a and IL12b was also downregulated by NiV V and W (Fig. 10 C-H). For all tested genes NiV W seemed to be a stronger inhibitor of transcription than NiV. In contrast, NDV V protein significantly induced the transcription of these immune genes.

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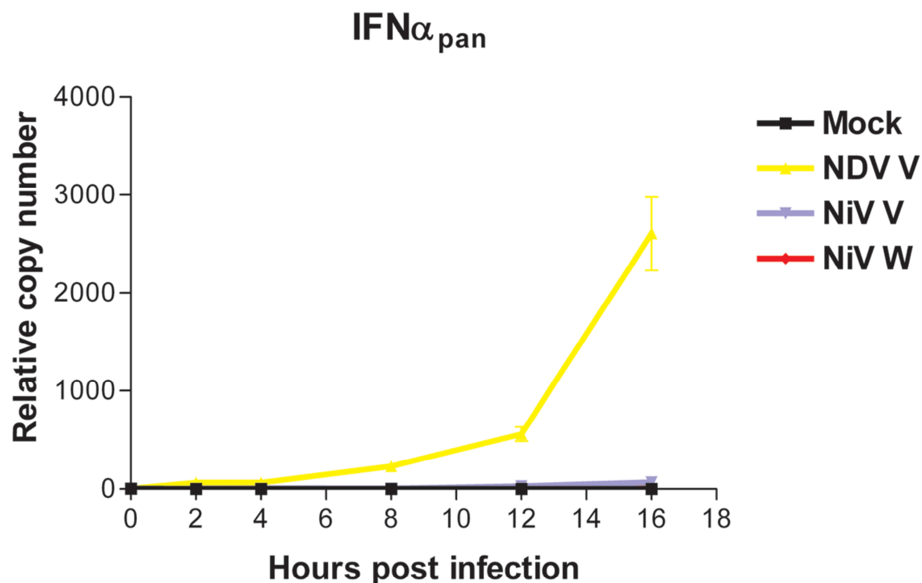

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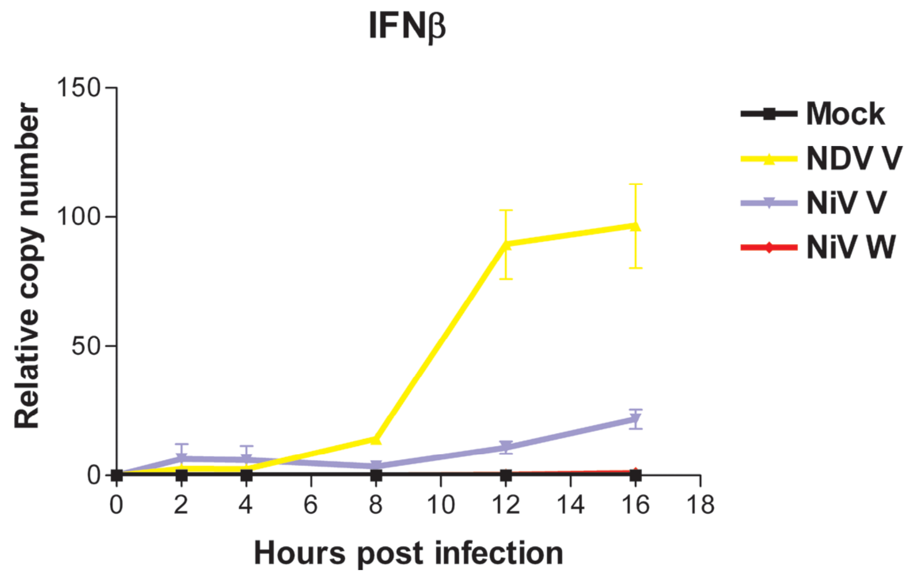

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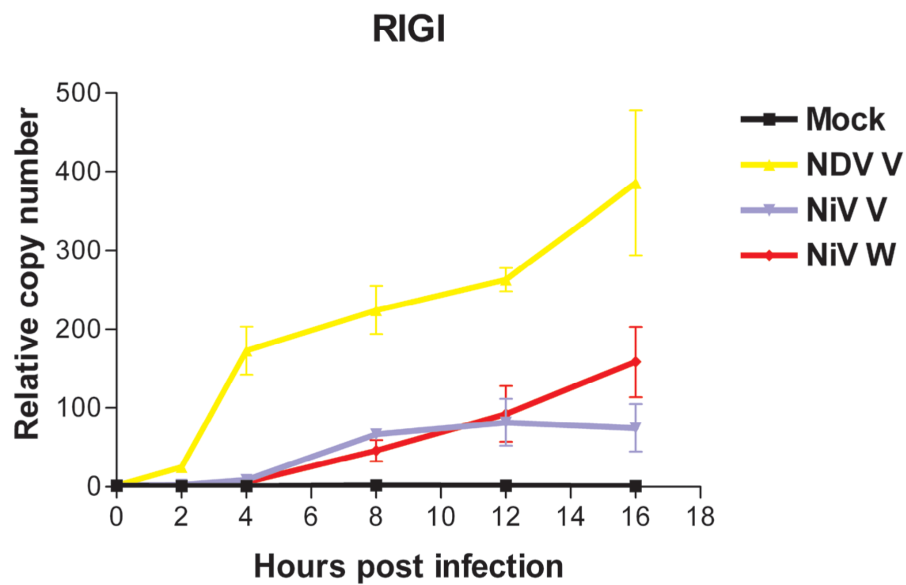

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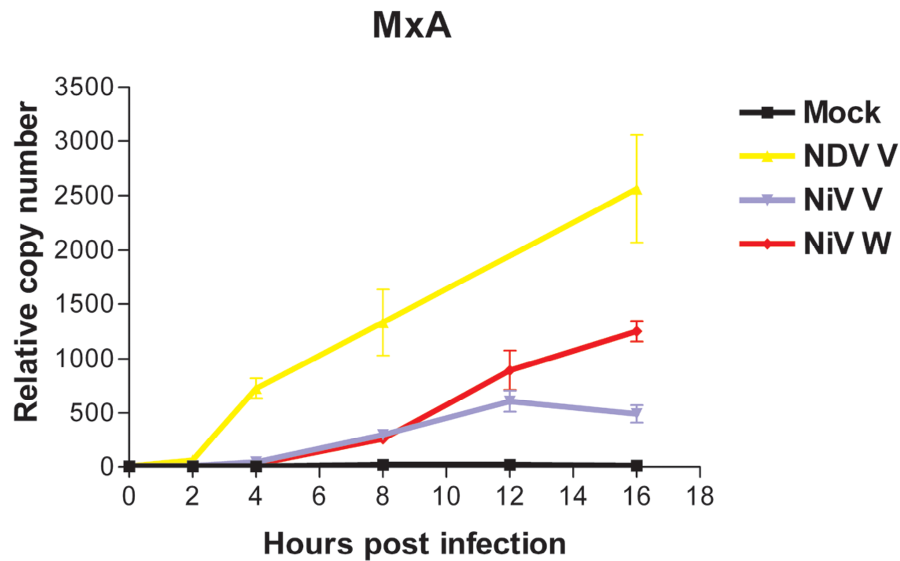

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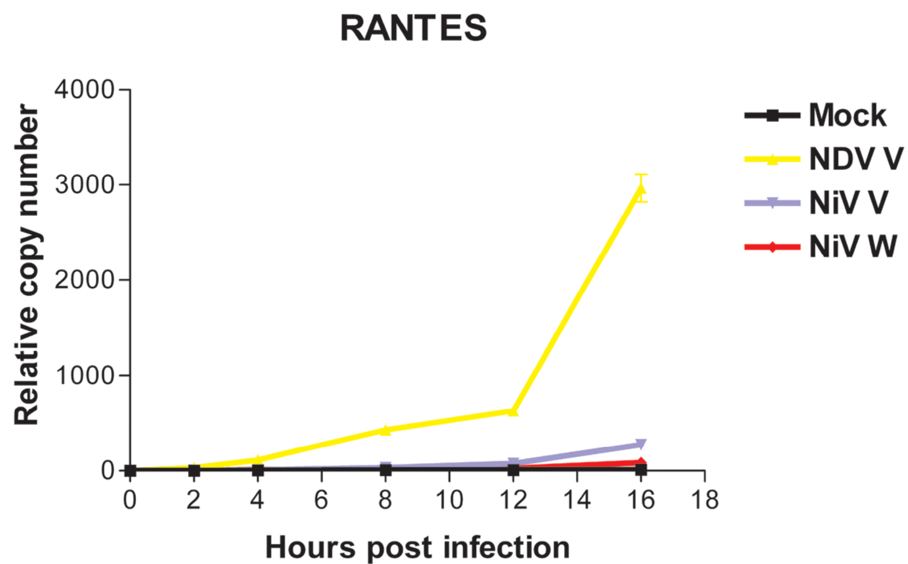

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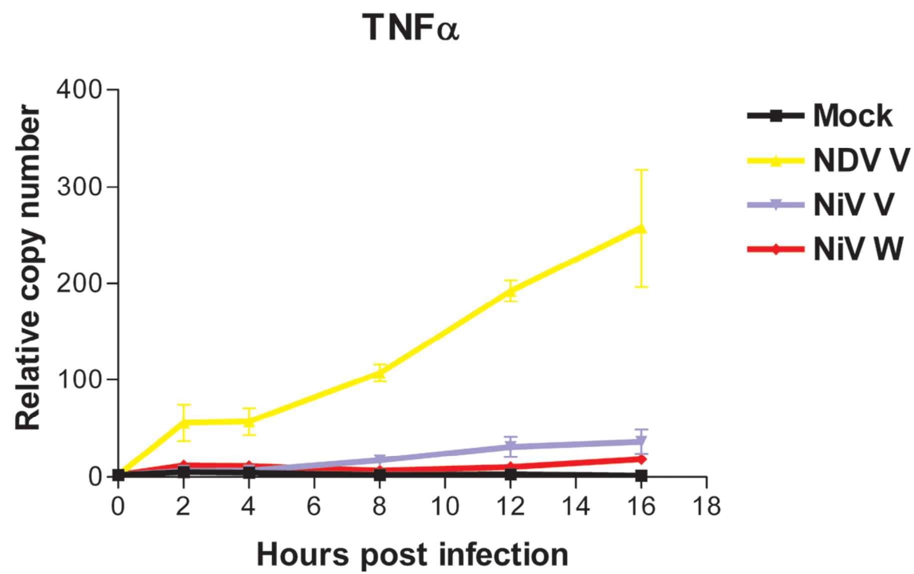

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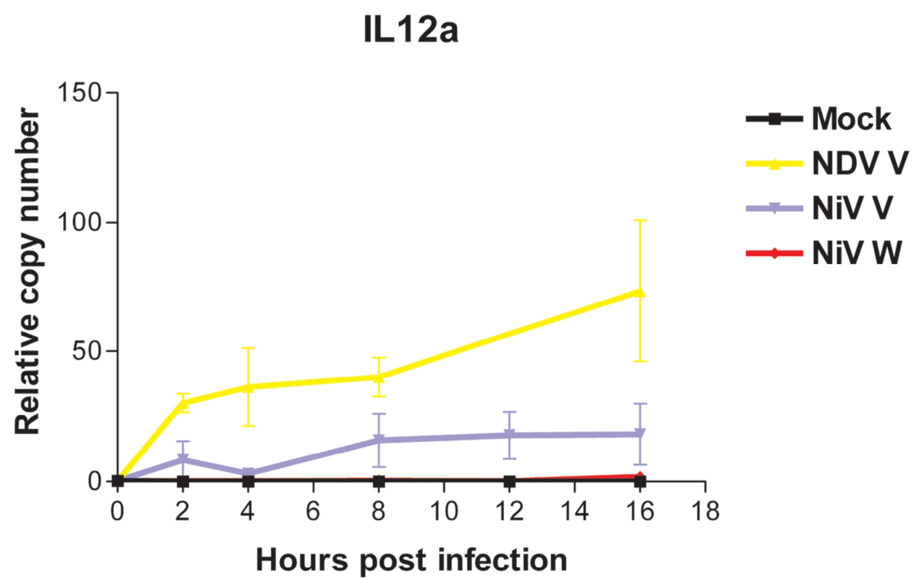

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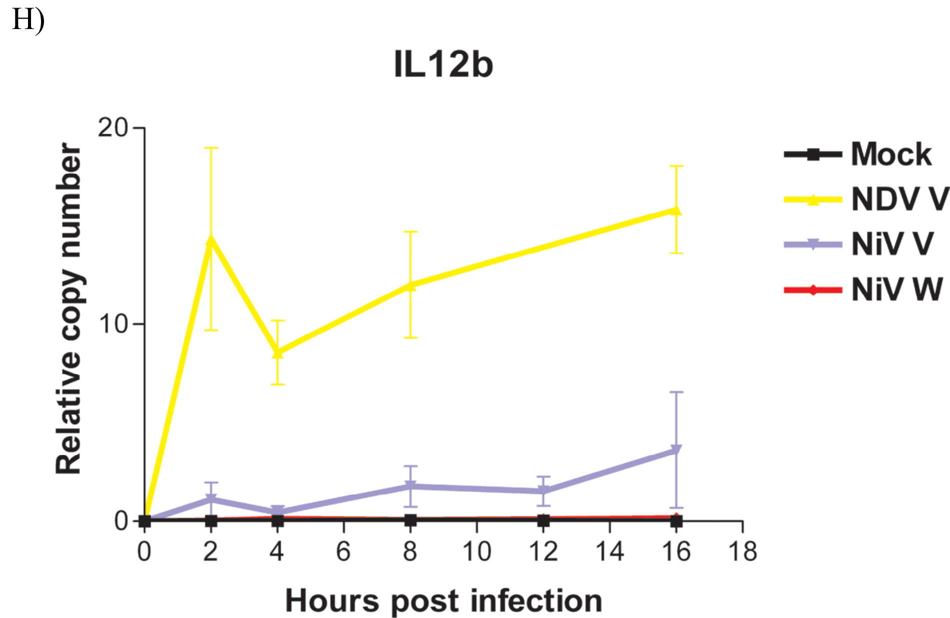

Fig. 10: NiV V and W protein potently downregulate transcription of important host immune genes.
Human DCs were either mock infected or infected with rNDV/NDV V, rNDV/NiV V rNDV/NiV W at an MOI of 2 (in triplicates). Cell lysates were collected at different time points after infection and RNAs were isolated and used to generate cDNAs to perform qRT-PCR. Values indicate changes in gene expression in mock infected and virus-infected DCs compared to expression of three housekeeping genes (actin, tubulin, rsp11). Error bars represent standard deviations for triplicate samples.

NiV V and W proteins were also strong inhibitors of the secretion of important immune-relevant proteins such as type I interferons, TNF-alpha, RANTES and IL12p70 (Fig. 11 A-E). The transcription of these genes was also downregulated (Fig. 10), which accounted for the fact that less protein was made and secreted. Moreover the secretion of MCP1-beta and IL-6 was downregulated in the presence of NiV V and W (Fig. 11 F and G). The transcription of these two genes was not tested by qRT-PCR. However, the result implied that a reduced secretion of both MCP1-beta and IL-6 genes was also caused

because of low protein levels. On the other hand, NDV V protein significantly induced the secretion of these factors. Consistent with the qRT-PCR data NiV W seemed to be a stronger inhibitor of protein secretion.

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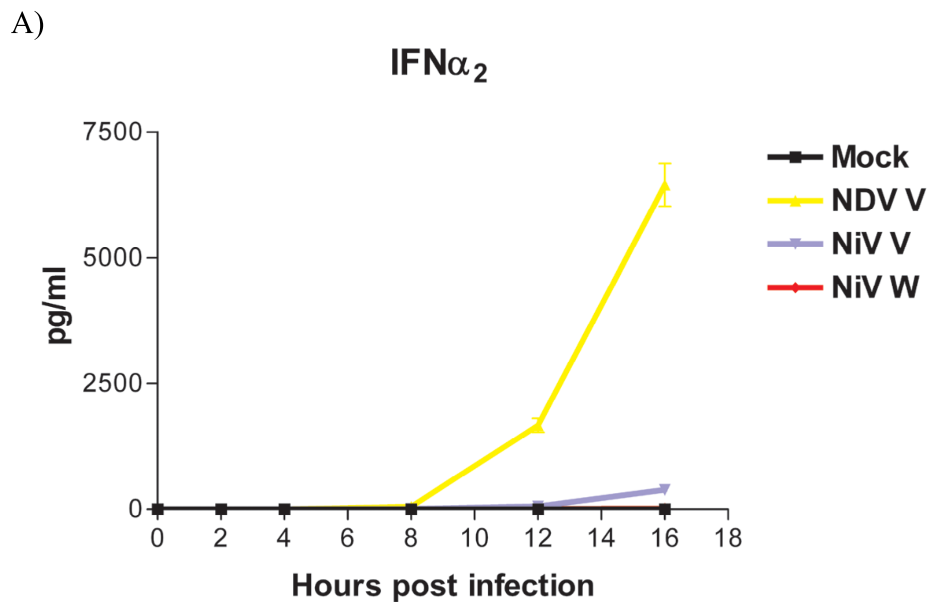

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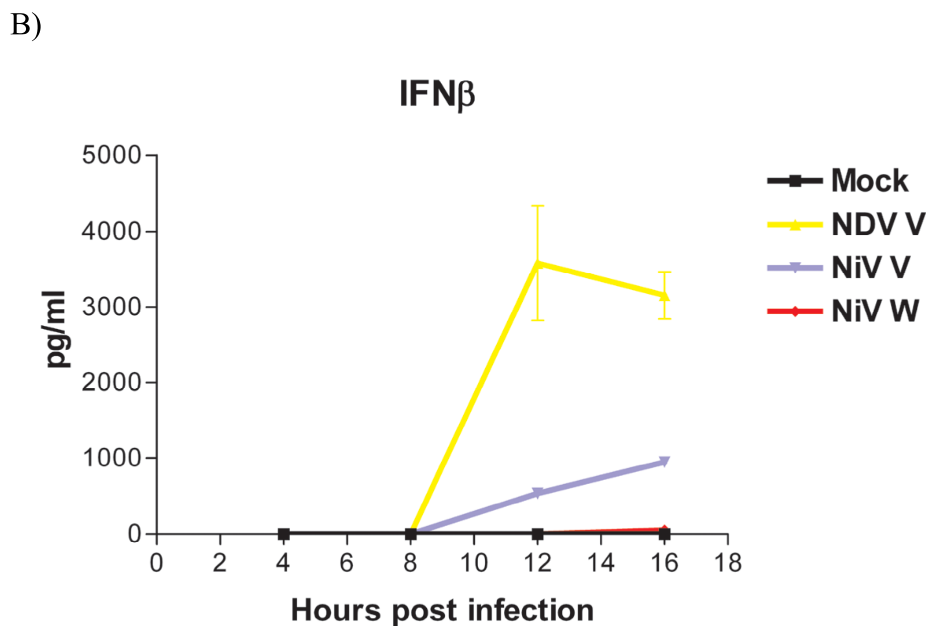

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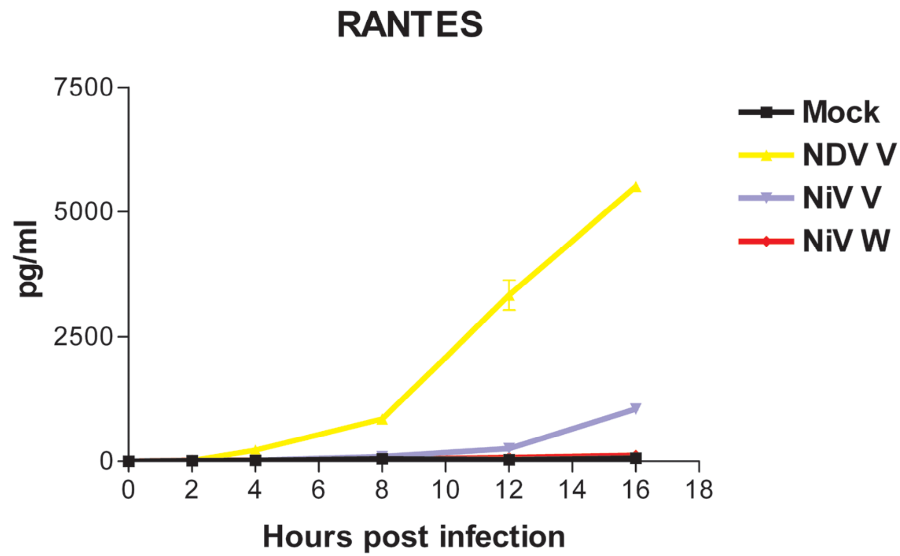

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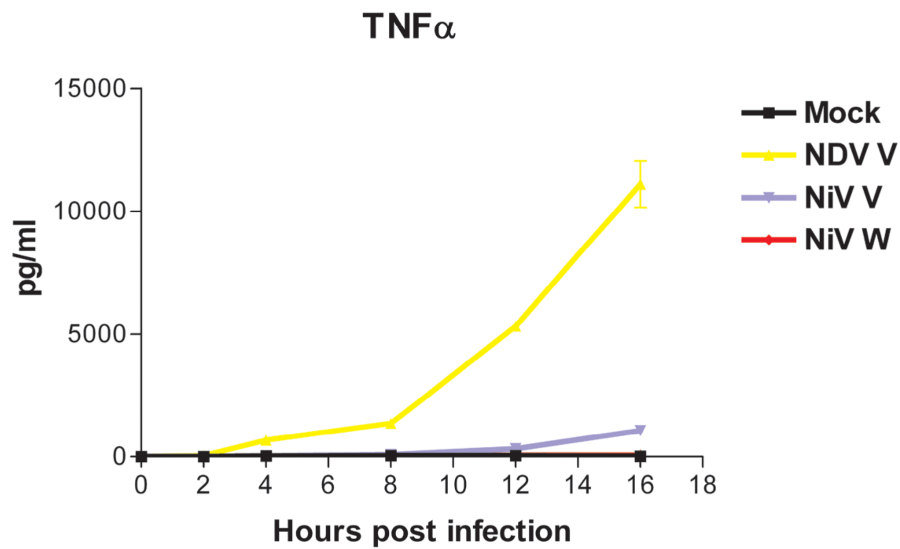

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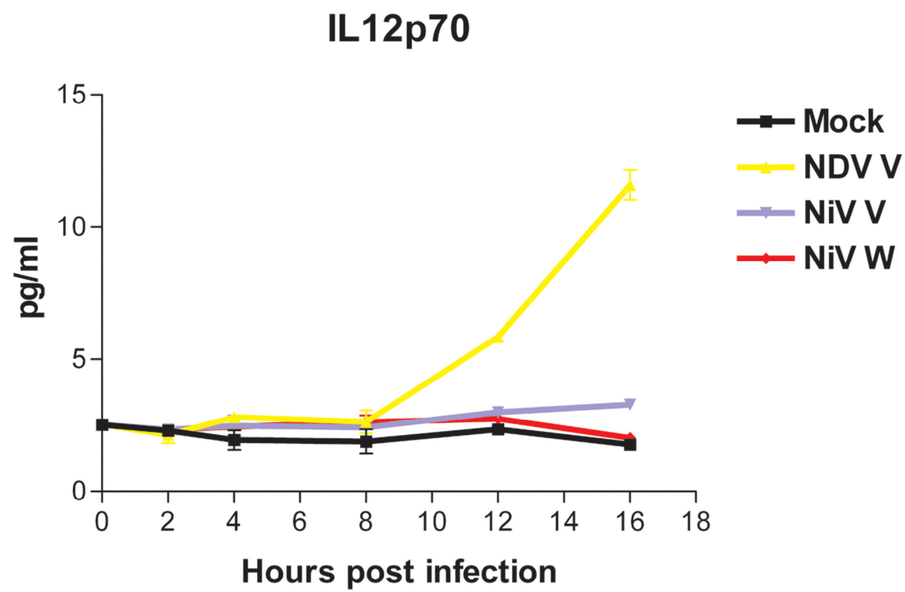

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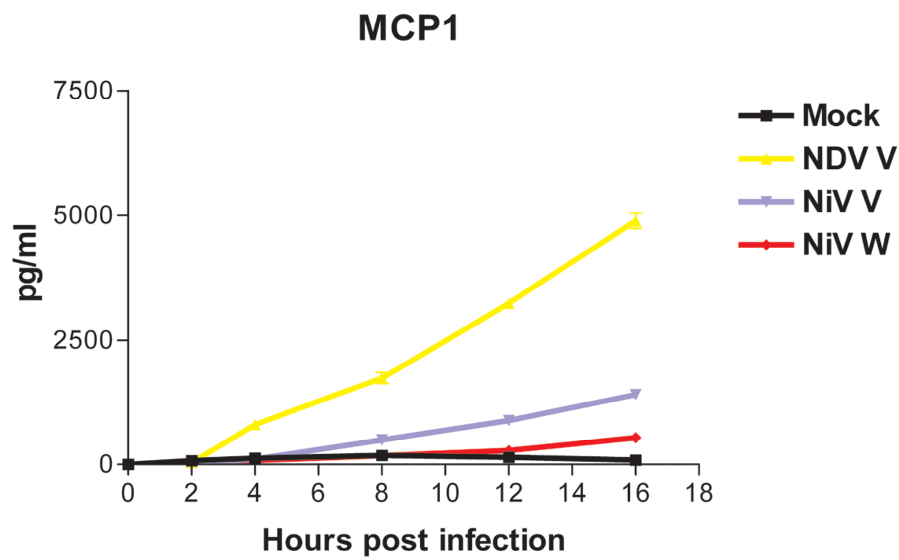

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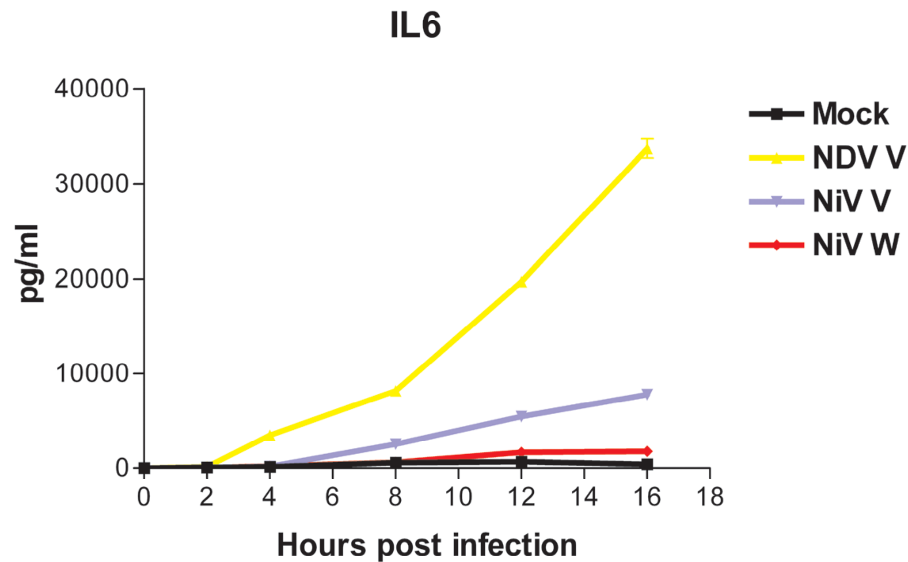

Fig. 11: NiV V and NiV W proteins potently downregulate the secretion of host immune proteins.

Human DCs were either mock infected or infected with rNDV/NDV V, rNDV/NiV V rNDV/NiV W at an MOI of 2 (in triplicates). Cell supernatants were collected at different times post infection and analyzed by ELISA for the presence of selected proteins. Error bars represent standard deviations for triplicate samples.

3.2 The effect of Nipah V and W proteins on viral replication

The C, V and W proteins of paramyxoviruses are known to play multiple roles in the viral life cycle, in IFN antagonism (Didcock *et al.*, 1999b; Goodbourn *et al.*, 2000; He *et al.*, 2002; Komatsu *et al.*, 2004; Lin *et al.*, 2005; Nanda & Baron, 2006; Ohno *et al.*, 2004; Park *et al.*, 2003b; Poole *et al.*, 2002; Rodriguez *et al.*, 2002; Shaffer *et al.*, 2003), in addition to regulation of viral transcription and replication both in vitro and in vivo (Bankamp *et al.*, 2005; Baron & Barrett, 2000; Curran *et al.*, 1991; Curran *et al.*, 1992; Horikami *et al.*, 1996; Kato *et al.*, 1997a; Kato *et al.*, 1997b; Lin *et al.*, 2005; Malur *et al.*, 2004; Parks *et al.*, 2006; Patterson *et al.*, 2000; Reutter *et al.*, 2001; Smallwood & Moyer, 2004; Tober *et al.*, 1998; Witko *et al.*, 2006).

Considering that details of henipavirus replication are not yet completely elucidated, it was of interest to analyze the roles of NiV V and W in this regard. The work of this chapter will show that both NiV proteins are able to inhibit NiV minigenome replication and furthermore interact with NiV N protein, an important component of the NiV polymerase. Hereby the first 50 amino acids of the N terminal domain of both V and W seem to be responsible for both inhibition and interaction. The results suggest a role for NiV V and W proteins in the regulation of NiV replication.

In order to answer the question whether NiV V or W protein may have a role in viral replication, a Nipah virus minigenome transfection system was used (Fig. 12) (Halpin *et al.*, 2004). A reporter gene GFP-CAT in negative sense was constructed. It was flanked by T7 promotor and terminator regions, which allowed the transcription by the T7

polymerase. The T7 polymerase was constitutively produced in BSR T7 cells. After cleavage of the 3’ end by an HDV ribozyme the GFP-CAT was transcribed in the presence of expression plasmids encoding the NiV polymerase N, P and L proteins. As a principle of the minireplicon system it has to be noted that paramyxovirus N, P and L proteins are necessary and sufficient for intracellular replication in vitro and in vivo (Knipe D.M., 2007) and this has been confirmed for henipaviruses by reverse genetics. GFP signal and most importantly CAT activity served as a signal that is directly proportional for viral transcription and replication. Thus, it was of interest to analyze the effect of NiV V and W proteins on the NiV minigenome activity. Constructs being used for the NiV minigenome system are summarized in Figure 13.

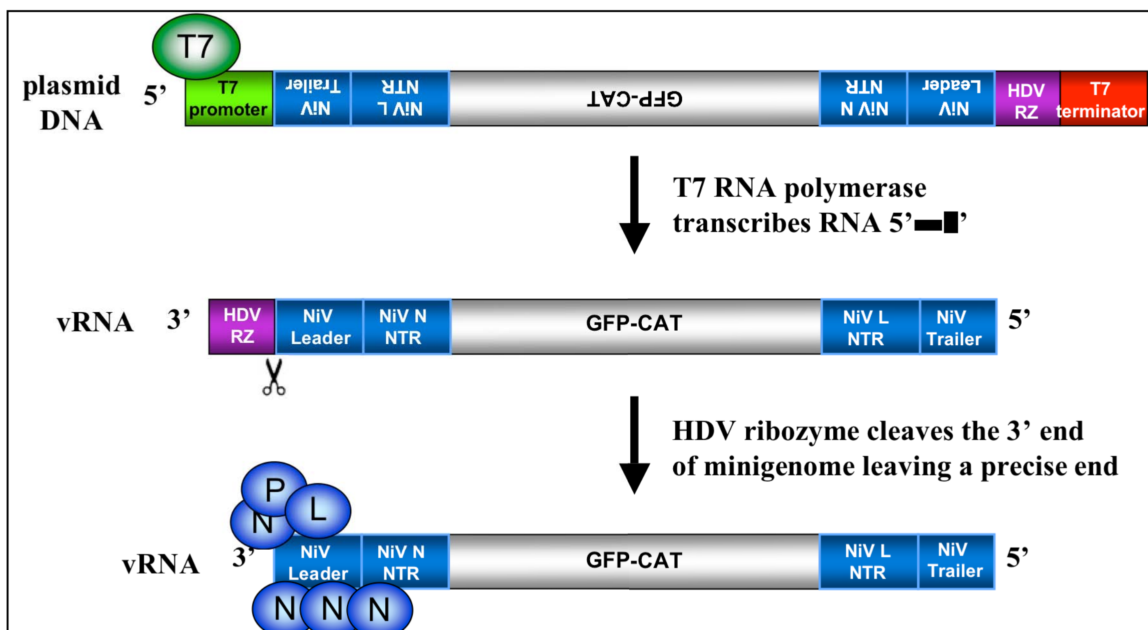

Fig. 12: Schematic representation of the NiV minigenome system.
The negative-sense GFP-CAT reporter gene (with NiV specific non-coding regions (NCR), Trailer regions, and HDV ribozyme site) is flanked by T7 promoter and T7 terminator. T7 polymerase, which is constitutively expressed by BSR T7 cells (baby hamster kidney cells), transcribes this plasmid DNA in 5'- 3' direction resulting in a RNA that mimics a vRNA. HDV ribozyme cleaves the 3' end of the minigenome leaving a precise end. NiV N, P and L proteins expressed from pTM1 expression plasmids are now able to bind to the 3' end and transcribe the GFP-CAT gene. This GFP-CAT mRNA is then translated into a protein (image kindly provided by Mike Ciancanelli).

In a first experiment BSR T7 cells were transfected with the plasmids of the NiV minigenome system and additional NiV V, W and C expression plasmids (Fig. 14). It should be mentioned that within the system the exact ratios of plasmids being transfected is crucial in order to guarantee CAT activity. As one can see both NiV V and W proteins reduced CAT activity whereas NiV W had a more potent inhibitory effect (more than 90% reduction of CAT activity). In contrast, there was no significant reduction of CAT activity in the presence of NiV C protein. This contradicts a recently published report that said that NiV C protein was able to inhibit NiV minigenome replication (Sleeman *et al.*, 2008). However, the results showed that NiV C only seemed to have an inhibitory effect when used at a high concentration of 2 µg. In contrast, here titration experiments revealed that an inhibitory effect was dose-dependent for all used NiV proteins with NiV W being the most potent inhibitor. The loss of complete inhibition of CAT activity occurred with NiV W at 31.3 ng, with NiV V at 250 ng and with NiV C at 1000 ng (data not shown). These results implied that NiV and W proteins (but not C protein) might indeed have a role in the regulation of viral replication.

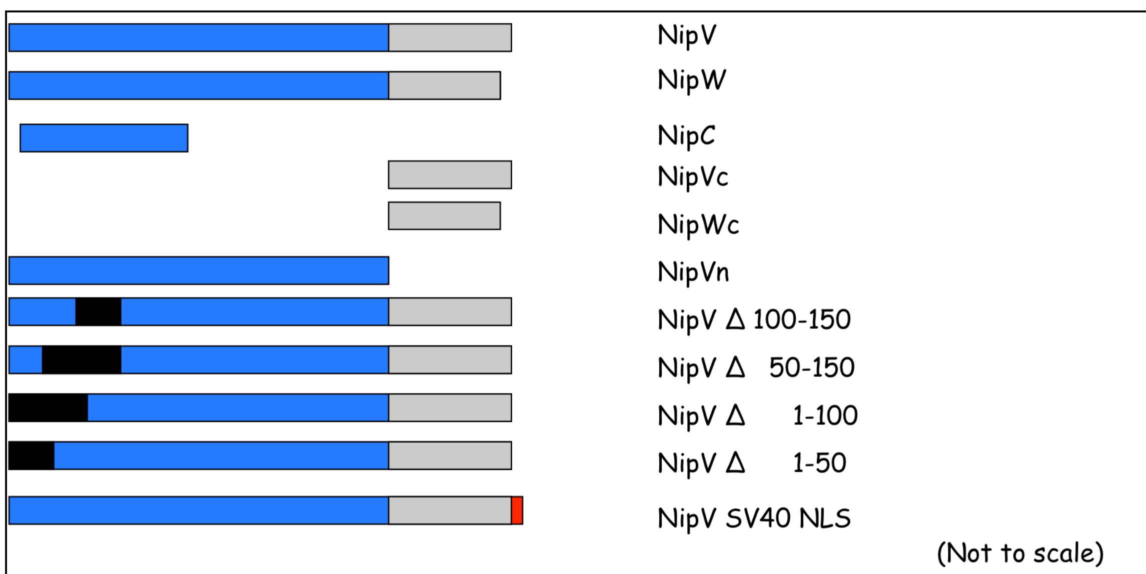

Fig. 13: NiV constructs.
The plasmids shown above were used for either NiV minigenome assay or Co-immunoprecipitations. Blue = N terminus, grey = C terminus, black = deleted regions, red = nuclear localization signal (NLS) of SV40. The N terminus is defined to be up to the editing site within the P ORF (amino acid 407). Full length Nip V consists of 456 amino acids.

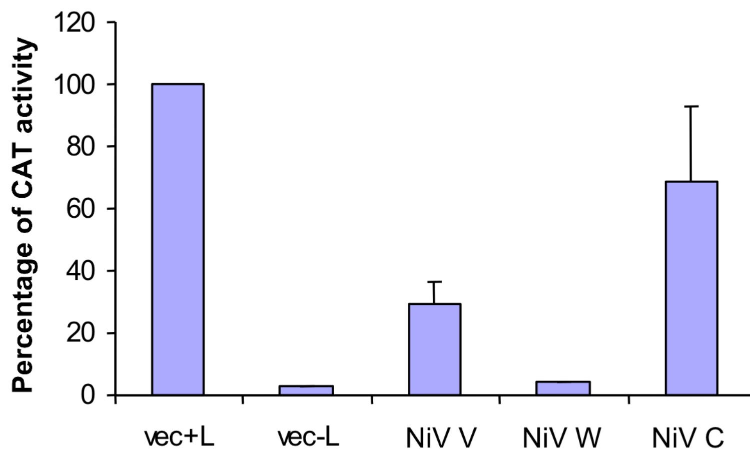

Fig. 14: NiV V and W proteins downregulate Nipah virus minigenome activity.
BSR T7 cells were transfected with the NiV GFP-CAT-Minigenome construct (3.5 µg) and constructs expressing NiV L (0.4 µg), N (0.75 µg) and P (0.05 µg). In addition cells were transfected with constructs expressing NiV V, W or C, respectively (0.5 µg). Cells were harvested 24 hours post transfection and subjected to CAT assay. Values were normalized to firefly luciferase activity. Error bars represent standard deviations for triplicate samples; vec+L (positive control), vec-L (negative control, cells did not receive the L-expressing construct).

In order to elucidate which part of NiV V and W protein was responsible for interfering with viral replication, the N terminus and C terminus of NiV V and NiV W were cloned into expression plasmids and used in the NiV minigenome system. An interesting feature of both NiV V and W proteins is that they share the same N terminus whereas their C terminus is unique because of RNA editing. Figure 15 shows that the N terminal region of both NiV V and NiV W (Nip Vn) seemed to be responsible for the inhibition of CAT activity. In contrast there was no significant reduction observed in the presence of the respective C termini (Nip Vc and Nip Wc).

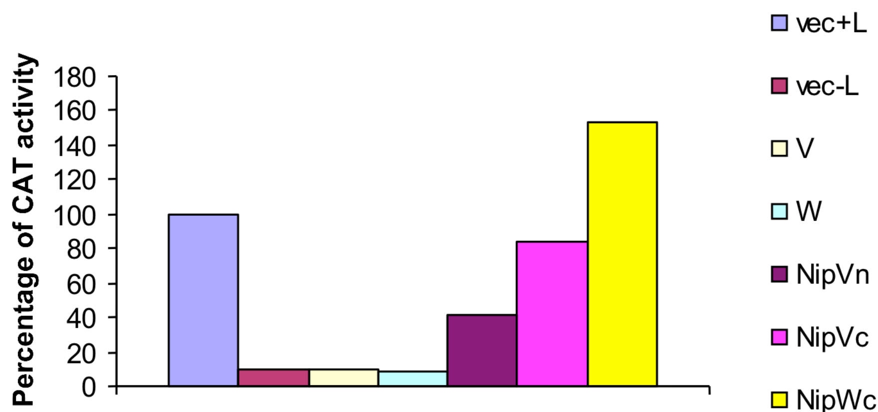

Fig. 15: The N-terminus of NiV V and W protein is responsible for downregulation of NiV minigenome activity.
BSR T7 cells were transfected with the NiV GFP-CAT-Minigenome construct (3.5 µg) and constructs expressing NiV L (0.4 µg), N (0.75 µg) and P (0.05 µg). In addition cells were transfected with constructs encoding NiV V and W or with constructs expressing the shared N terminus of V and W (Vn) or the unique C terminus of V and W (Vc and Wc), respectively (2 µg). Cells were harvested 24 hours post transfection and subjected to CAT assay. Values were normalized to Firefly Luciferase numbers. Results represent one of three individually performed experiments; vec+L (positive control), vec-L (negative control, cells did not receive the L-expressing construct).

Based on the NiV minigenome data it was of interest whether NiV V and W proteins might interact with components of the NiV polymerase. The NiV polymerase complex is composed of NiV L, N and P proteins (Halpin *et al.*, 2004). Hereby the focus was on interactions with NiV N protein. Constructs being used for co-immunoprecipitations are summarized in Figure 13. A co-immunoprecipitation experiment in 293T cells showed that both NiV V and W proteins interacted with NiV N protein (Fig. 16). The interaction between NiV P and N served as a positive control. Moreover NiV N did not interact with NiV C supporting the NiV minigenome results (data not shown).

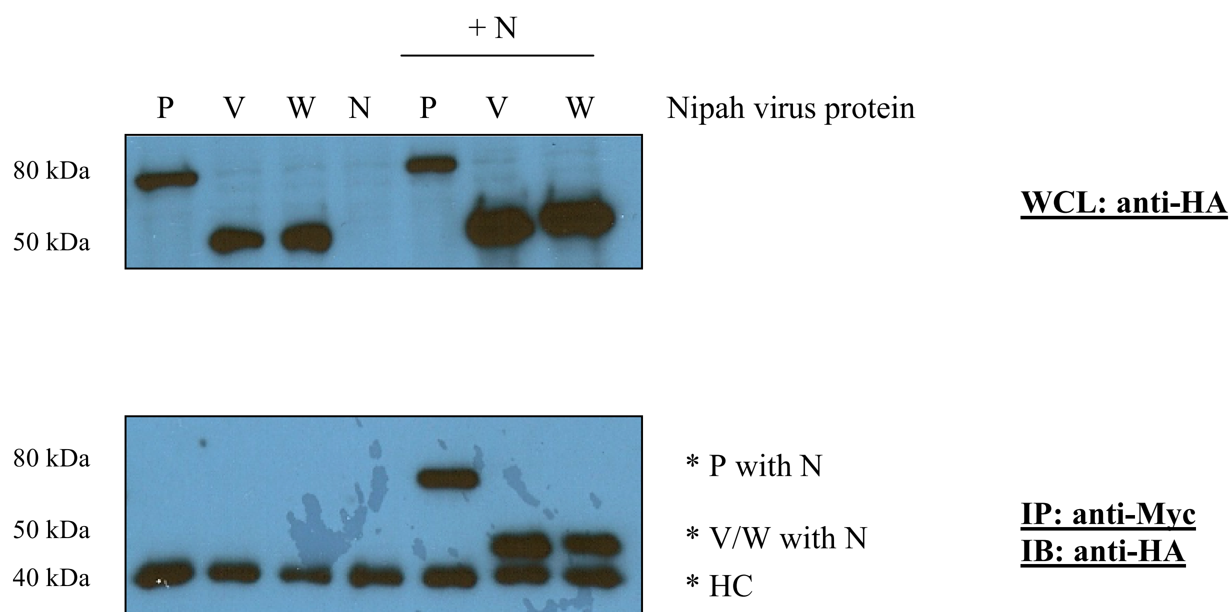

Fig. 16: NiV V and W proteins interact with NiV N protein.
293T cells were transfected with transfected with constructs expressing HA-tagged NiV P, V or W with or without Myc-tagged NiV N (each 2 µg). 24 hours post transfection cells were harvested and incubated with anti-Myc antibody for immunopreciptiation (IP). Immunoblotting (IB) occurred with anti-HA antibody; WCL (whole cell lysates), HC (heavy chain), molecular weight (kDa).

The results of the NiV minigenome experiments suggested that the inhibitory domain was localized within the N terminus of both NiV V and W proteins, so several N terminal mutants of NiV V were cloned and used for co-immunoprecipitation experiments (Fig. 17). Since the N terminus of both NiV V and NiV W is identical, NiV V was used for the generation of the new constructs. As seen before, full length and N terminal NiV V protein interacted with NiV N protein (Fig. 17). Furthermore, interaction between NiV N and NiV V deletion mutants NiV V Δ 100-150 and NiV V Δ 50-100 was detectable (Fig. 17). In contrast, NiV V Δ 1-100 and NiV V Δ1-50 lacked the ability to bind to NiV N (Fig. 17). This lead to the conclusion that amino acids 1-50 of both NiV V and NiV W

proteins seemed to be important for binding to NiV N protein. Interestingly, when a NLS from SV40 was attached to the NiV V protein, it was still able to bind to NiV N. This showed that the subcellular localization of NiV V and NiV W did not play a role regarding their binding capacity to NiV N. Both cytoplasmic and nuclear NiV V protein was able to interact with NiV N. This observation correlated also with the fact that NiV W predominantly locates to the nucleus (Shaw *et al.*, 2005).

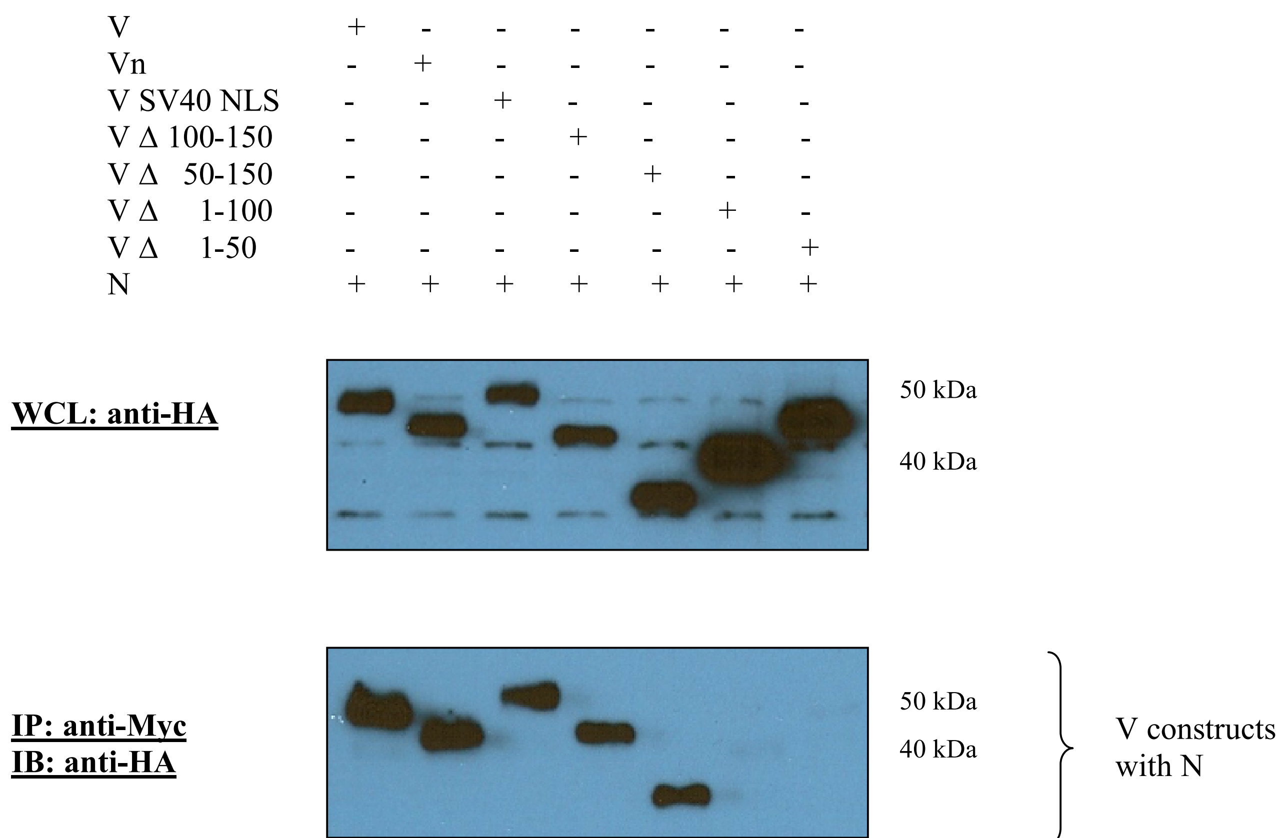

Fig. 17: N-terminal amino acids 1-50 of NiV V and W are important for binding to NiV N.
293T cells were transfected with different HA-tagged NiV V expression constructs in the presence of Myc-tagged NiV N (each 2 µg). 24 hours post transfection cells were harvested and incubated with anti-Myc antibody for immunopreciptiation (IP). Immunoblotting (IB) occurred with anti-HA antibody; WCL (whole cell lysates), molecular weight (kDa).

Furthermore, the results of another co-immunoprecipitation experiment implied that NiV V and W might interact with both NiV N and NiV P in a tertiary complex (Fig. 18). When increasing amounts NiV P, V, W and influenza virus NP proteins (as a negative control) are co-expressed with proteins of the whole NiV polymerase machinery, it was observed that NiV V interacted with both NiV N and NiV P at the same time (Fig. 18). The same was true for NiV W (Fig. 18). In contrast, influenza virus NP protein did not interact with NiV N or NiV P. This suggested, that the observed interactions between the NiV proteins were specific. One may also speculate whether NiV L protein might be another binding partner for the tertiary complex. This would imply a quaternary complex between NiV L, N, P and NiV V or NiV W. Here, we were not able to test for it since the detection of NiV L protein is difficult and there is currently no functional antibody against NiV L available.

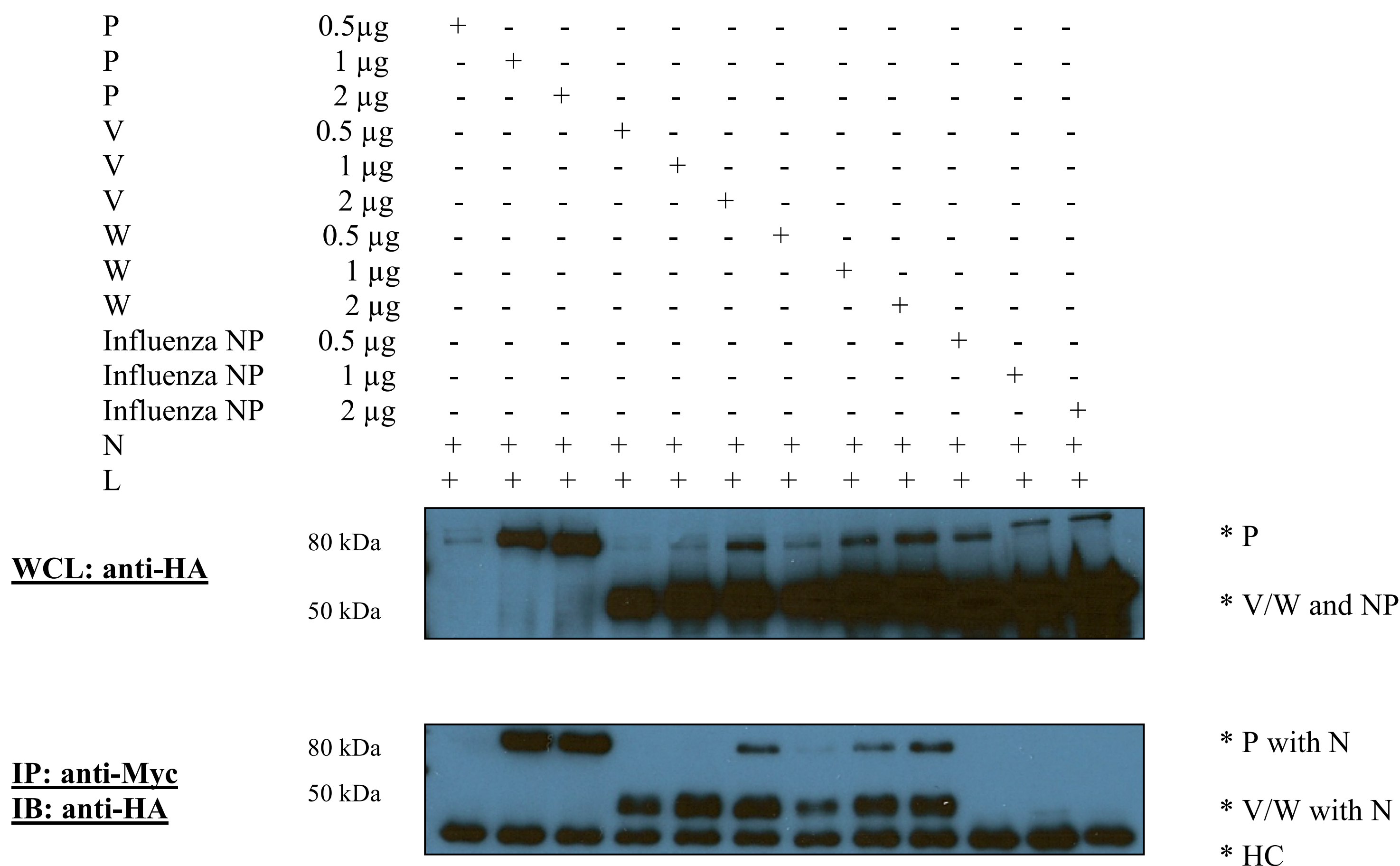

Fig. 18: Potential complex formation of NiV V and NiV W with NiV P and NiV N. 293T cells were transfected with constructs expressing HA-tagged NiV P, V, W or Influenza NP in increasing concentrations (0.5, 1 and 2 µg) in the presence of Myc-tagged NiV N (0.75 µg), NiV L (0.4 µg) and un-tagged NiV P (0.05 µg). 24 hours post transfection cells were harvested and incubated with anti-Myc antibody for immunoprecipitiation (IP). Immunoblotting (IB) occurred with anti-HA antibody; WCL (whole cell lysates), HC (heavy chain), molecular weight (kDa).

In addition, immunofluorescence experiments were performed in order to support the results of the NiV minigenome and co-immunoprecipitation experiments (Fig. 19). NiV N co-localized with NiV P, NiV V and NiV W, respectively. Interestingly, NiV N protein

was found together with NiV P protein in O-ring like structures in the cytoplasm (top panel). For instance, this phenomenon was previously observed for human respiratory syncytial virus (Carromeu *et al.*, 2007) and suggested that the viral polymerase forms specific replication “bodies”. In correlation with the previous data both cytoplasmic NiV V (middle panel) and nuclear NiV W protein (bottom panel) colocalize with NiV N protein in the cytoplasm and nucleus, respectively.

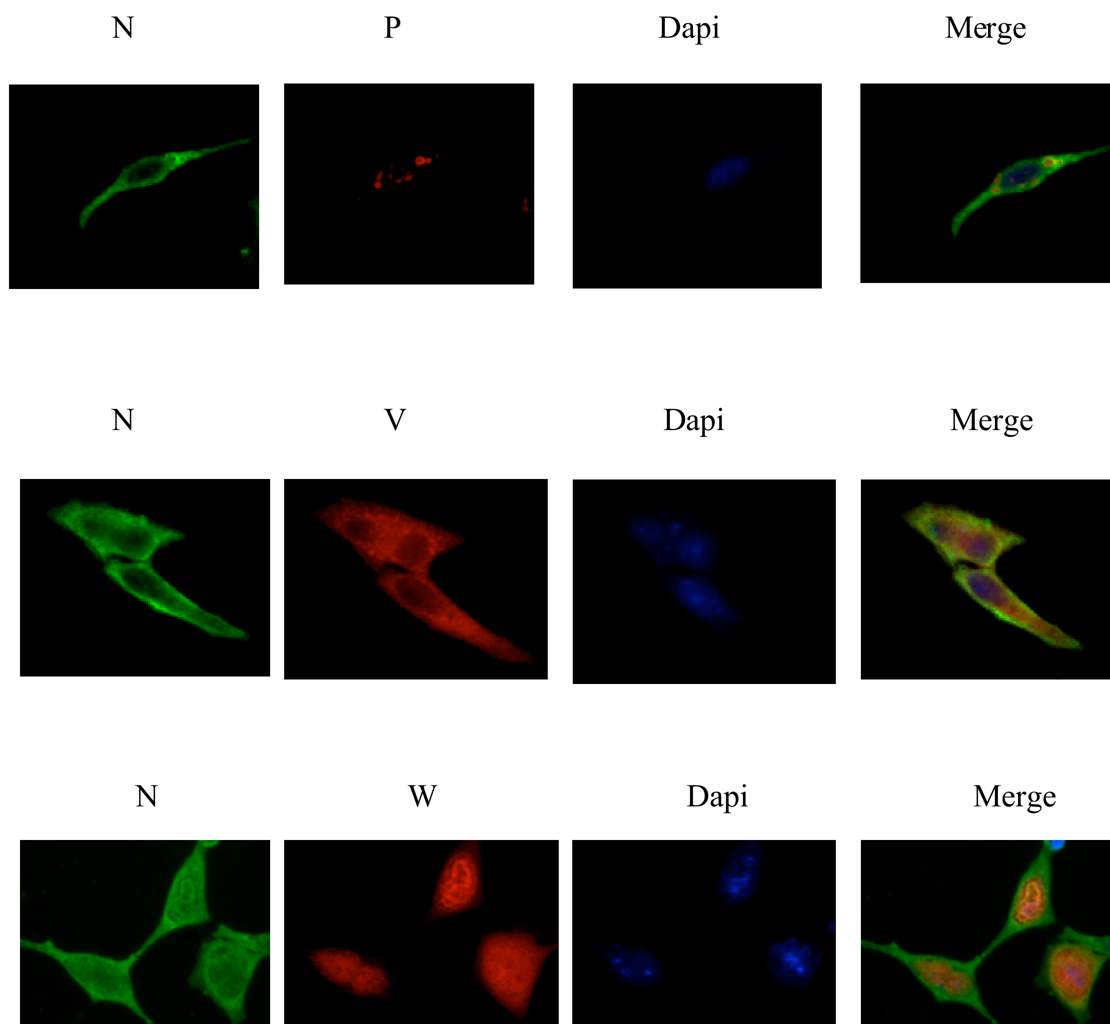

Fig. 19: NiV V and NiV W proteins colocalize with NiV N protein, respectively. HeLa cells were transfected with pCAGGS plasmids encoding HA-tagged NiV P, V or W in the presence of Myc-tagged NiV N (each 2 µg). Approximately 24 hours post transfection cells were fixed, permeabilized and stained against HA- and Myc tag.

4. Discussion

4.1 Characterization of Nipah virus V and W proteins on the human host immune response

4.1.1 Characterization of recombinant Newcastle disease virus expressing Nipah virus V and W proteins

This part is about the characterization of rNDVs expressing NiV V and W proteins. Recombinant NDVs expressing the respective ORFs were generated. Since it was of specific interest to corroborate the IFN antagonism of both NiV V and W proteins in infected human cells, it was necessary to include a control virus that would not cause a suppression of the host immune response. For this purpose rNDV expressing NDV V protein was used. Although the insert location of the NDV V ORF differs from the one for NiV V or W ORFs, the usage of this virus was justified by the observation that it behaved like wildtype NDV. For this and the following chapters we compared rNDVs expressing NiV proteins to rNDV expressing the avian NDV V protein.

The results of the growth curve experiments confirmed the hypothesis (Fig. 5). Recombinant NDVs expressing NiV V and W proteins grew to higher titers in human A549 cells compared to rNDV expressing the avian NDV V protein. Thus, NiV V and W proteins enhanced the replication capacity of the avian backbone virus. Efficient viral replication kinetics is evidence for viral pathogenicity, so the results strongly suggest an important role for NiV V and W proteins in the human host. It has been seen in various outbreaks with a high mortality rate that Nipah virus represents a considerable threat for humans (Ali *et al.*, 2001; Chua *et al.*, 2000a; Chua *et al.*, 2000b; Sahani *et al.*, 2001). In

contrast, because of their avian origin all rNDVs displayed similar growth kinetics in CEF cells. Similar growth kinetics for all rNDVs could also be observed in Vero cells. These cells are not able to produce interferons and hence not able to influence viral replication. This emphasized the intriguing possibility that NiV V and W proteins might be the main contributors to the specifically high lethality observed in humans.

Moreover the localization of NiV V and W proteins within the cell is different (Fig. 6). It has been previously shown in transfection experiments that the V protein is mainly localized in the cytoplasm and the W protein in the nucleus (Shaw *et al.*, 2005; Shaw *et al.*, 2004). The same observation was made in A549 cells that were infected with the respective recombinant NDVs. NiV might be a potent virus because its proteins are able to counteract the host immune response from different cell compartments. This possibility has already been confirmed in experiments where NiV V and W proteins were transiently expressed. Experiments presented further below will address this question in the context of a viral infection.

4.1.2 Nipah virus V and W proteins potently downregulate the interferon system in human A549 cells

The work of this chapter focuses on the impact of NiV V and W proteins on the human host immune response during a viral infection. As opposed to an infection with Nipah virus, the proteins of interest were produced by recombinant NDVs. Infected A549 cells were tested for changes in their immune response at the level of transcription and translation of selected important immune genes/proteins. Via this selection it was possible to examine the effect of the NiV proteins on different steps of the host IFN

system. Briefly, both NiV V and W proteins exhibited a profound inhibitory effect on the expression of multiple genes/proteins of the IFN system compared to NDV V, a strong inducer of the host immune response.

Quantitative RT-PCR experiments showed that transcription of both IFN-alpha and IFN-beta genes were strongly downregulated in the presence of NiV V and W proteins over time (Fig. 7 B and C). Furthermore mRNA expression levels of important IFN-stimulated genes were reduced. Type I interferons play an important role for the activation of ISGs. The binding to the interferon receptor on the cell surface triggers the intracellular IFN signaling pathway -mainly the JAK-STAT pathway-which eventually induces the expression of a large number of ISGs. The ISGs set up an antiviral, antiproliferative and immunoregulatory state in the host cells. Gene transcription and production of IFN-dependent proteins is only possible if transcription factor STAT1 translocates in its dimerized version into the nucleus. Quantitative RT-PCR showed that transcription of STAT1 was downregulated by both NiV V and W (Fig. 7 D). A STAT1-inhibitory effect has previously been shown for SV5, human metapneumovirus and Hepatitis B virus (Didcock *et al.*, 1999a; Dinwiddie & Harrod, 2008; Wu *et al.*, 2007; Young *et al.*, 2000) and most importantly also for Nipah virus V and W proteins (Ludlow *et al.*, 2008; Shaw *et al.*, 2004). This suggests, that NiV V and W proteins might reduce overall STAT1 protein expression and additionally block the activity of STAT1. The mRNA levels of other transcriptional activators such as IRF1, IRF7 and NFkappaB1 (p50) were also reduced in the presence of NiV V and W proteins (Fig. 7 E, F and G). It has been shown before that NiV W protein allows for the inhibition of both virus- and Toll-like receptor 3-triggered signaling pathways by reducing overall levels of phosphorylated IRF-3. This

suggests, that NiV W might not only inhibit the transcription of IRFs but also its activity. However, this is not true for NiV V, which is only able to inhibit the RIG-I-dependent pathway (Shaw *et al.*, 2005). Normally the stimulation of IFN-beta gene transcription by viral infection or dsRNA is mediated by a tertiary complex-enhanceosome consisting of NFkappaB, IRFs, activated protein 1(AP-1), JUN and the high mobility protein HMG-1, which are recruited to the virus responsive elements (VRE) of the IFN-beta promotor. This enhanceosome further recruits histone acetyl transferases (HAT) and the CREB binding protein (CREB). The expression of the IFN-alpha gene subtypes is also regulated at the transcriptional level. IRF-1, IRF-3 and IRF-7 together with histone transacetylases are part of the transcriptionally active IFN-alpha enhanceosome. Thus, while the activation of IFN-beta gene transcription is regulated by both NFkappaB and IRF-3, activation of the IFN-alpha genes depends mostly on IRF (Paun & Pitha, 2007). However, the fact that mRNA expression levels of IRFs and NFkappaB were reduced in the presence of both NiV V and W proteins, implied that the formation of the IFN-enhanceosome complex was disrupted. One would expect a diminished number of functional enhanceosomes that are present in the cell resulting in less transcription of the genes for IFNs and inflammatory cytokines. This hypothesis was supported by the result demonstrating a reduction of type I interferons levels by NiV V and almost complete abrogation by NiV W at a transcriptional level (Fig. 7 B and C). NiV V and W proteins also downregulated the transcription of other selected ISGs such as ISG54, ISG56, RANTES, RIG-I, MxA, TNF-alpha, IL6 and CCL3 (MIP1-alpha) (Fig. 7 H-O). The P54 and P56 proteins (transcribed from genes ISG54 and 56) are inhibitors of cellular (and viral) translation (Daffis *et al.*, 2007; Hui *et al.*, 2003; Sen & Peters, 2007; Sen & Sarkar,

2007). An inhibition of these proteins (or their gene transcription) implies to be an advantage for NiV in order to guarantee its replication. RIG-I is essential for the recognition of viruses such as NDV, VSV, SeV, Ebola virus, Lassa virus, Nipah virus, influenza virus or JEV via their 5' phosphate termini (Habjan *et al.*, 2008; Kato *et al.*, 2006; Kumagai *et al.*, 2008). Henipavirus V protein has also been shown to interact with viral sensor MDA-5 thereby preventing dsRNA signaling (Andrejeva *et al.*, 2004; Childs *et al.*, 2007). MxA proteins are important factors involved in the antiviral response by inhibition of viral replication (Kim & Maniatis, 1997; Mundt, 2007). RANTES is a cytokine that functions as a chemoattractant for blood monocytes, memory T helper cells and eosinophils and has been shown to be important for the antiviral immune response (Liao *et al.*, 2008; Pulendran *et al.*, 2008; Zhao *et al.*, 2007). The inhibition of important cyto- and chemokines may be beneficial for viral growth. TNF-alpha displays a repertoire of different functions. It has been shown to promote apoptosis, necrosis but also cell proliferation, differentiation and inflammation (Dutta *et al.*, 2006). IL-6 is a major hallmark for an ongoing viral infection and exerts important functions in the inflammatory response (Svitek *et al.*, 2008). Likewise, CCL3 (MIP-1) is involved in the acute inflammatory state and also in the recruitment and activation of polymorphonuclear leukocytes (Stuart *et al.*, 2008; Wolpe *et al.*, 1988). Inhibition of these factors may contribute to the survival of the virus at the cost of the host. Worth mentioning is the observation that NiV W protein seemed to be a stronger inhibitor of transcription than NiV V for all tested genes. In contrast, NDV V protein induced an immune response by upregulating the transcription of all these genes.

Quantitative RT-PCR results were confirmed by the data of a specific immune Microarray that was performed for rNDV-infected A549 cells (Fig. 8 and Table 2). Hereby, 1,070 immune genes were tested for up- or downregulation during viral infection. Figure 8 showed that NDV V protein caused a greater upregulation of gene transcription compared to NiV and W. NiV W seemed to downregulate mRNA expression more potently than NiV V, which is in agreement with the previous qRT-PCR data. Table 2 enlists all the genes that were significantly downregulated by both NiV V and W proteins compared to NDV V. A ≥ 50% downregulation was considered to be significant. Genes that were previously tested by qRT-PCR, were also found to be downregulated in the presence of NiV V or W in the Microarray. In this regard both Microarray and qRT-PCR data match for NFkappaB1 (p50), STAT1, CCL3 (MIP1-alpha), RANTES, IRF1, IFN-beta, IRF7 and ISG56 (Table 2, green shaded boxes). In the Microarray transcription of IL-6 was not significantly reduced in the presence of NiV V and W, however qRT-PCR also measured comparatively low relative copy numbers for IL-6 (Fig. 7 N). For this reason qRT-PCR data for IL-6 may not be significant. The same may be suggested for TNF-alpha because obtained relative copy numbers were in the same range as seen for IL-6 (Fig. 7 M). There was no data available for transcription of TNF-alpha and IFN-alpha in the Microarray due to low quality or low signal intensities of the samples. Moreover, ISG54, RIG-I and MxA were not included in the array. However, Microarray data for the other genes demonstrated their downregulation at the level of transcription, which was also seen by qRT-PCR. Furthermore the transcription of numerous other genes was inhibited in the presence of NiV and W (Table 2). The functions of their proteins are versatile. Essential transcription factors such as ATF3,

JUNB and members of the NFkappaB family were affected. It has been shown that these factors are targets for modulation by measles virus, coxsackievirus, varicella-zostervirus and rotavirus (El Mjiyad *et al.*, 2007; Helin *et al.*, 2002; Holloway & Coulson, 2006; Hwang *et al.*, 2007; Indoh *et al.*, 2007). Stress- or apoptosis-related genes were also included. IAP1 inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 (Rothe *et al.*, 1995; Van Eden *et al.*, 2004). GADD34 is a member of a group of genes whose transcription levels are increased following stressful growth arrest conditions (Hollander *et al.*, 1997). Caspase 4 plays a central role in the execution-phase of cell apoptosis (Kamada *et al.*, 1997). MCL1 belongs to the Bcl-2 family. Its longer isoform 1 enhances cell survival by inhibiting apoptosis while the alternatively spliced shorter isoform 2 promotes apoptosis and is death inducing (Yang-Yen, 2006). PDCD1LG2 (B7DC) is a programmed cell death 1 ligand 2 and stimulates T cell proliferation and IFN-gamma production in dendritic cells (Tseng *et al.*, 2001). Interferon-induced genes such as IFITM1 and IFITM2-IFITM3 were also included. Moreover the gene transcription of chemokine CCL20 and cytokines CCL2 and CXCL12 was downregulated in the presence of NiV V and W. The mRNA expression levels of ectoenzymes or enzymes such as CD38, SOD2, GN6ST and PARG1 were also low. Transcription of important factors involved in signal transduction pathways, FIP2 (NEMO2), IkappaB-alpha, SOCS1, C1S, MAP3K8 and PIAS-1, were affected by NiV and W. Likewise factors with a role in plasmin formation (PLAUR, PAI1), cell adhesion (CEACAM1-CEACAM2), transport (TAP1) and cell growth (IGFBP6) were downregulated. Transcriptional expression of B2M, which associates with MHC class I heavy chain, was reduced as well. Table 2 emphasized that NiV V and W downregulated

the transcription of genes whose gene products have manifold functions in the host immune system. Table 2 also shows that some of the enlisted genes were more potently downregulated by NiV W than by V. For this comparison the values for V and W were compared and a ≥ 1.7 fold difference was considered as significant. Miltenyi Biotech used this specific value in order to determine significance for the Microarray. To be consistent, the same value was applied here (see Materials and Methods for further explanation). Interestingly, NiV W seemed to be a stronger inhibitor for the transcription of the IFN-beta gene and of transcriptional activators such as ATF3, JUNB and NFkappaB1 and 2. The mRNA levels of cytokines CCL2, CCL3 and RANTES were also more potently reduced in the presence of NiV W than V. The same was observed for IAP1, IkappaB-alpha and ISG56. This may point to a more virulent role for NiV W compared to V, when expressed from rNDV.

The effect of NiV V and W was also determined at the level of translation. Hereby, the focus was on two essential IFN-stimulated genes RIG-I and MxA. The protein expression of both RIG-I and MxA was significantly reduced in the presence of NiV and W compared to NDV V (Fig. 9). This result was in agreement with the observation that NiV V and W also inhibited the transcription of these genes (Fig. 7 K and L).

In conclusion, NiV V and W proteins had an inhibitory effect on the transcription of important immune genes that are involved in both IFN-induction and IFN-signaling during viral infection. This suggests that NiV may indirectly cause a block of different pathways that would normally contribute to the clearance of a viral infection. Interestingly, NiV W protein caused a greater inhibition of transcription than NiV V. A

possible explanation for this observation may be the nuclear localization of NiV W. This guarantees a close contact to the host DNA and may facilitate the inhibition of gene transcription. However, it is not known yet which NiV W-specific determinants might contribute to this phenotype. The inhibition examined here was mainly constituted of downregulation of transcription/translation. NiV V and W proteins, when transiently expressed, have been previously shown to inhibit the interferon system by preventing IFN-induced STAT phosphorylation and nuclear translocation (Rodriguez *et al.*, 2002; Shaw *et al.*, 2005; Shaw *et al.*, 2004). It has also been discovered that the IFN antagonism is encoded in the common N terminus (amino acids 5-150) of NiV P, V and W (Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). NiV V or NiV W protein could possibly sequester important transcriptional factors. It has been previously shown that NiV V is able to form high-molecular complexes with transcriptional activators STAT1 and 2 (Rodriguez *et al.*, 2002; Shaw *et al.*, 2004). Preliminary data imply that NiV V and W proteins sequester NFkappaB in the cytoplasm, which prevents its translocation to the nucleus (data not shown). This has been previously shown for varicella-zoster virus (El Mjiyad *et al.*, 2007). Interference with the NFkappaB pathway has also been shown for numerous viruses such as HIV-1, respiratory syncytial virus, SARS coronavirus and VSV (Hiscott *et al.*, 2006). Another possibility would be an inhibition of the host translation machinery. It has been shown for measles virus that its N protein is able to inhibit host translation by binding to eIF3-p40 (Sato *et al.*, 2007). The cytoplasmic localization of NiV V may be of advantage for a potential translational block. There is a need for further investigations in order to explore these possibilities for NiV V and W proteins.

4.1.3 Nipah virus V and W proteins potently downregulate the interferon system in human dendritic cells

Dendritic cells have an important role within the host immune response. They have very good antigen presentation properties and are able to activate B- and T-lymphocytes during (viral) infections. These specialized immune cells are then responsible for the clearance of pathogens in the body. However, DCs also provide a safe haven for viruses and it has been shown that Ebola virus and dengue virus are able to infect DCs (Bray & Geisbert, 2005; Fuller *et al.*, 2007; Kyle *et al.*, 2007; Martinez *et al.*, 2007; Nightingale *et al.*, 2008). Nipah virus primarily infects endothelial cell, however to date nothing is known about Nipah virus and its impact on dendritic cells.

Here, isolated human monocyte-derived DCs were infected with rNDV expressing NiV V and W protein, respectively. A comprehensive analysis of human DC activation was conducted using quantitative real-time PCR. A similar approach was previously published for the study of influenza virus NS1 protein (Fernandez-Sesma *et al.*, 2006).

It was shown that NiV V and W proteins strongly downregulated the transcription of type I interferons and IFN-stimulated genes and also the secretion of interferons and important cytokines and chemokines over time (Fig. 10 and 11). In contrast, NDV V induced the transcription and secretion of these factors. A number of studies have demonstrated a requirement for type I IFNs for optimal maturation of myeloid DCs (Fujii *et al.*, 2004; Honda *et al.*, 2003; Lopez *et al.*, 2004). However, transcription and secretion of both IFN-alpha and IFN-beta was strongly reduced in the presence of NiV V or NiV W (Fig. 10 and Fig. 11, both A and B). Quantitative RT-PCR measured not one specific but all 13 IFN-alpha subtypes therefore resulting in higher relative copy numbers compared to IFN-

beta. This relation might have been different if only one specific IFN-alpha subtype (e.g. IFN-alpha 2) had been analyzed. Moreover NiV V and W caused a reduction of RIG-I mRNA expression levels (Fig. 10 C). From several studies it is known that RIG-I is essential for antiviral responses to a specific set of RNA viruses such as flaviviruses, paramyxoviruses, orthomyxoviruses and rhabdoviruses (Kato *et al.*, 2006; Sumpter *et al.*, 2005; Yoneyama *et al.*, 2004). In contrast to the TLR system in plasmacytoid DCs, conventional DCs use RIG-I as one of the main sensor of viral RNA. Therefore a reduction of RIG-I seems to be advantageous for the virus. The transcription of MxA, another important antiviral factor, was also downregulated in the presence of NiV V and W (Fig. 10 D). Likewise, transcription and secretion of IL-12 was reduced (Fig. 10 G and H, Fig. 11 E). IL-12 plays an important role in the differentiation of naïve T cells into Th1 cells and activation/stimulation of T cells and natural killer cells. Moreover it stimulates the production of IFN-gamma. IL-12 is a heterodimeric cytokine, encoded by two separate genes, IL-12a (p35) and IL-12b (p40). The active heterodimer is formed after protein synthesis. It was observed that transcription of both IL-12 a and b genes was downregulated and there was also a reduced secretion of the active heterodimer IL-12p70. Relative copy numbers and levels of secreted IL-12 were consistently lower compared to those of the other tested genes/proteins. TNF-alpha and IL-6, two representative proinflammatory cytokines, were significantly less released into the culture supernatant of DCs in the presence of NiV V and W compared to NDV V (Fig. 11 D and G). Likewise, the secreted levels of MCP1 (recruits monocytes and memory T cells), and RANTES (chemotactic for T cells, eosinophils and basophils) were significantly decreased (Fig. 11 F and C). Quantitative RT-PCR showed a reduction in transcription

for all tested genes (excluding MCP-1 and IL-6 which were only analyzed by ELISA) in the presence of NiV V and W (Fig. 10). The reason for a low secretion of these factors was due to a low transcription of their genes as seen by qRT-PCR. This transcriptional inhibition might have accounted for a reduced protein production and secretion. Hereby, NiV W protein was a stronger inhibitor of transcription and secretion compared to NiV V. This observation was also made for rNDV-infected A549 cells (Chapter III B). DCs and A549 cells represent different cell types and experiments were carried out with a different amount of cells, therefore one cannot compare the results in detail such as relative copy numbers of transcribed genes. However, it can be proposed that both NiV V and W protein have a potent inhibitory role on the human host immune system.

In conclusion both NiV proteins were able to attenuate the function of human dendritic cells. Not only the function of DCs in innate immunity was compromised by abrogating the type I interferon response, but also the establishment of the link to adaptive immunity was severely disturbed by inhibition of cytokines and chemokines that are important for stimulation of T cells. Co-culture experiments with T cells in the presence of DCs might further allow for examination of the activation state of T cells (e.g. by looking for the presence of T cell specific surface molecules that are expressed after stimulation or IFN-gamma production). A previous study has reported the presence of NiV antigens in cells of the human lung, liver, spleen, kidney and CNS (Hooper *et al.*, 2001), but it has to be demonstrated yet that DCs are infected in vivo.

4.2 The effect of Nipah V and W proteins on viral replication

Non-structural proteins are encoded by many paramyxoviruses and it is generally accepted that they have multiple roles in the viral life cycle such as regulation of viral transcription/replication or modulation of the host immune response (Horikami *et al.*, 1997; Horikami *et al.*, 1996; Takeuchi *et al.*, 2001). NiV V and W proteins have been shown to significantly downregulate the host IFN system (Rodriguez *et al.*, 2002; Shaw *et al.*, 2005). In order to address the question whether NiV V and W proteins also possess the ability to regulate viral replication, a plasmid-based reverse genetic system was used. A similar approach has been recently published for Nipah virus (Sleeman *et al.*, 2008) and there are other reports for several other paramyxoviruses including measles virus (Witko *et al.*, 2006), human parainfluenzavirus 3 (Durbin *et al.*, 1997), Sendai virus (Tapparel *et al.*, 1997) and respiratory syncytial virus (Atreya *et al.*, 1998). Figure 14 demonstrated that NiV V and W protein inhibit CAT expression from the NiV minigenome with NiV W being a stronger inhibitor than V. NiV C did not inhibit minigenome activity. This was in contrast to a recently published report by Sleeman et. al in which they claim that NiV C did have an inhibitory effect on the NiV minireplicon (Sleeman *et al.*, 2008). This was in agreement with other published studies suggesting that paramyxovirus C protein inhibits viral replication (Curran *et al.*, 1992; Smallwood & Moyer, 2004). However, since NiV C is expressed from another ORF, this protein does not share any sequence similarity with NiV V or W. Especially it has to be noted that the N terminus of NiV C differs from the N terminal regions of NiV V and W. This suggests, that NiV C might act on viral replication via another mechanism compared to

NiV V and W. Moreover, Sleeman et al. based their interpretations on experiments that were performed with high concentrations of NiV C plasmid (2µg). The high concentration of NiV C in the cell might have oversaturated the minireplicon system resulting in a reduction of CAT activity. This might explain the discrepancy between their study and the here presented study. Moreover, the N terminal domains of NiV and W seemed to be important for inhibition of the NiV minireplicon (Fig. 15). The fact that the N terminus of NiV V and W is identical suggests that downregulation of CAT activity was specific to NiV V and W. In this context it should be noted, that the same inhibition could also be observed in the presence of additional NiV P protein. P also shares the same N terminus with NiV V and W and the observed inhibition of CAT activity was likely to be a feature the N terminus. Therefore, the hypothesis was that V and W might interact and block the function of the NiV polymerase. Indeed both NiV proteins bound to NiV N protein, which constitutes together with L and P the NiV polymerase complex (Fig. 16). Further experiments with N terminal deletion mutants revealed that the first 50 amino acids were important for the binding of NiV V (or NiV W) to NiV N (Fig. 17). Interestingly, NiV V with an artificially inserted NLS was still able to bind to NiV N. This implied that interaction of NiV V and W with NiV N occurred regardless their localization in the cell. RNA replication occurs in the cytoplasm, but NiV W protein is localized in the nucleus. As a possibility NiV W might downregulate viral replication by sequestration of NiV N or other components of the NiV polymerase into the nucleus thereby preventing its access to the viral RNA. On the other hand NiV V resides in the cytoplasm and may be directly involved in the regulation of viral replication. This would support other reports because the V proteins of several paramyxoviruses have been

shown to be able to regulate the replication of the viral genome. Recombinant paramyxoviruses lacking V proteins, including measles virus (Tober *et al.*, 1998), Sendai virus (Kato *et al.*, 1997b) and rinderpest virus (Baron & Barrett, 2000) produced increased levels of genomic RNA, mRNA and viral proteins. Moreover, the V protein of SV5 inhibited viral transcription and replication (Lin *et al.*, 2005). The exact mechanism by which V controls viral replication is not completely understood yet but direct V-N interactions (Svenda *et al.*, 2002; Sweetman *et al.*, 2001) and V-RNA (Parks *et al.*, 2006) interactions have been postulated. Besides a V-N interaction, V-N-P or even V-N-P-L interactions may be possible. It was also suggested that NiV V or W might form a tertiary complex with both NiV N and NiV P (Fig. 18). Immunofluorescence experiments showed that NiV N, which normally resides in the cytoplasm, co-localizes with NiV P, V or W, respectively (Fig. 19). NiV N can be observed together with P or V in the cytoplasm and with W in the nucleus. Thus, NiV W may have a dominant effect on N and therefore able to draw it into the nucleus. The mechanism by which this occurs remains to be resolved.

In conclusion this work presents evidence that both NiV V and NiV W proteins have a role in the regulation of the viral genome. Thus, besides being virulence factors for the host immune system, these proteins might also accomplish essential regulatory tasks for the virus itself. However, questions about exact mechanisms, timing and complex structural interactions still have to be further investigated.

Overall this work demonstrated that NiV V and W are important proteins for NiV because of various reasons. Both proteins serve as crucial virulence factors by inhibiting the host immune system and as regulators of viral replication itself. In addition to RNA

editing and alternative ORFs, many viral proteins have shown to be multifunctional in order to compensate for the small viral genome. There is still much to learn about how Nipah virus is able to counteract the host immune response and to ensure its replication at a molecular level, but further investigations might provide important details.

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6. Appendix

6.1 Summary

The work of the previous chapters describes the role of Nipah virus V and W proteins regarding their role in interferon antagonism and regulation of viral replication.

Previous publications have shown that NiV encodes IFN antagonist activity in its V, W and C protein (Park *et al.*, 2003b; Rodriguez *et al.*, 2002). In order to study the effect of both NiV proteins in the context of a virus infection, recombinant Newcastle disease viruses expressing NiV V or NiV W were constructed. As a control virus served rNDV expressing NDV V proteins, which behaved like wildtype NDV. Growth kinetic experiments demonstrated that rNDVs expressing NiV V or W grew to higher titers than rNDV expressing NDV V in human A549 cells. This result suggested that both NiV V and W were able to render the avian virus, which normally does not replicate well in human cells, into a better growing virus. This hypothesis was supported by the fact that all rNDVs grew similarly in avian DF1 or Vero cells. When rNDV-infected A549 cells were specifically stained for NiV V or W protein it was observed that V is localized in the cytoplasm whereas W could be predominantly found in the nucleus. This observation was in agreement with previous studies reporting a NES for NiV V and a NLS for NiV W (Rodriguez *et al.*, 2004; Shaw *et al.*, 2005). The specific localization of each NiV protein has also been shown to contribute to different functions in terms of IFN antagonism (Shaw *et al.*, 2005).

Here, NiV V and W proteins caused a severe attenuation of the immune response in rNDV-infected human A549 and dendritic cells. The transcription of type I interferons and ISGs was significantly downregulated in the presence of NiV V and W proteins. As a

consequence of the transcriptional block, there was also an inhibition at the level of translation (as seen for A549 cells) and the secretion of IFNs and cytokines/chemokines (as seen for DCs). In contrast, NDV V protein induced a host immune response. Both NiV V and W also displayed a strong inhibitory effect on the function DCs. DCs represent a very important cell class because they link the innate immune response to the adaptive immune response (Banchereau & Steinman, 1998). By downregulating the production and secretion of important cytokines/chemokines that are important for the activation of B and T lymphocytes, NiV V and W were able to disrupt that link. Interestingly, NiV W seemed to be a stronger inhibitor than NiV V in both A549 cells and DCs. Overall, it was demonstrated that NiV V and W were able to prevent the induction of the innate and adaptive host immune response cascade by inhibiting the transcription of immune genes in DCs and A549 cells.

The second part of this work addressed the question whether NiV V and W proteins have a regulatory role in viral replication. This has been previously reported for Nipah virus itself (Sleeman *et al.*, 2008) and other negative-stranded viruses (Atreya *et al.*, 1998; Horikami *et al.*, 1996; Witko *et al.*, 2006). In order to study the ability of the V and W proteins of NiV to regulate viral transcription and/or replication, an existing NiV minireplicon assay was used (Halpin *et al.*, 2004). Here, it was shown that NiV V and W (but not C) proteins significantly downregulated NiV minireplicon activity. The common N terminal region was shown to harbor the inhibitory activity. Co-immunoprecipitation experiments showed that both NiV V and W (but not C) were able to interact with NiV N, one component of the NiV polymerase. This result was supported by immunofluorescence experiments that revealed co-localization of NiV N with V and W.

The binding of NiV V or W to NiV N occurred via their N terminus and more specifically amino acids 1-50. This suggested that V and W might inhibit viral replication by interacting with the viral polymerase resulting in a loss of function. Exact mechanisms still have to be elucidated.

6.2 Zusammenfassung

In dieser Arbeit wurde auf die Rolle von Nipah Virus V und W Proteinen bezueglich deren Rolle im Interferon-Antagonismus und Regulation der viralen Replikation untersuch.

In vergangenen Veroeffentlichungen wurde gezeigt, dass NiV seine interferon-antagonistische Aktivitaet in dessen V, W und C Proteinen kodiert (Park *et al.*, 2003b; Rodriguez *et al.*, 2002). Um den Effekt beider NiV Proteine im Rahmen einer Virusinfektion zu untersuchen, wurden rekombinante NDVs, welche entweder NiV V oder W exprimieren, konstruiert. Als Kontrollvirus diente ein rNDV, der das NDV V Protein exprimiert. Dabei verhaelt sich der rekombinante Virus wie NDV-Wildtyp. Kinetische Wachstumsexperimente in A549 Zellen zeigten, dass rNDVs, die NiV V oder W exprimieren, zu hoeheren Titern wuchsen als rNDV, der NDV V exprimiert. Dieses Ergebnis deutete darauf hin, dass sowohl NiV V als auch NiV W im Stande waren, das Vogelvirus NDV, welcher normalerweise schlecht in humanen Zellen repliziert, zu einem besseren Wachstum anzuregen. Diese Hypothese wurde durch die Tatsache unterstuetzt, dass alle rNDVs ein recht aehnliches Wachstumsverhalten in Vogelzellen (DF1 Zellen) und Vero Zellen, die kein IFN synthetisieren koennen, aufwiesen. Ausserdem wurden

rNDV-infizierte A549 Zellen spezifisch gegen NiV V oder W Protein angefaerbt und es konnte beobachtet werden, dass V im Zytoplasma und W ueberwiegend im Nukleus der Zelle lokalisiert ist. Diese Beobachtung stimmte mit vorhergehenden Studien ueberein, die von ein Nukleus-Exportsignal (NES) fuer NiV V und ein Nukleus-Lokalisationsignal (NLS) fuer NiV W berichteten (Rodriguez *et al.*, 2004; Shaw *et al.*, 2005). Es wurde gezeigt, dass diese unterschiedliche, jedoch spezifische Lokalisation der NiV Proteine zu verschiedenen Funktionen bezueglich des Interferon-Antagonismus beitraegt (Shaw *et al.*, 2005).

NiV V und W Proteine fuehrten zu einer heftiger Attenuation der Immunantwort in rNDV-infizierten humanen A549 und dendritischen Zellen. In der Anwesenheit von NiV V und W Proteinen wurde die Transkription der Typ 1-Interferonen und ISGs signifikant herunterreguliert. Als Konsequenz des transkriptionellen Blocks konnte auch eine Inhibition auf translationalem Niveau (zu sehen in A459 Zellen) und eine Inhibition der Sekretion von IFNs und Zytokinen/ Chemokinen (zu sehen in DCs) verzeichnet werden. NDV V Protein hingegen provozierte eine Immunantwort im Wirt. Sowohl NiV V als auch W zeigten auch einen starken inhibitorischen Wirkung auf die Funktion von DCs. DCs repraesentieren einen sehr wichtigen Zelltyp, da sie eine Verbindung zwischen der angeborenen und erworbenen Immunantwort herstellen (Banchereau & Steinman, 1998). Durch die Herunterregulierung der Produktion und Sekretion wichtiger Zytokine/Chemokine, die fuer die Aktivierung von B- und T-Lymphozyten wichtig sind, waren NiV V und W faehig, diese Verbindung zu zerstoeren. Interessanterweise schien NiV W in A549 Zellen ein viel staerkerer Inhibitor zu sein als NiV V. Insgesamt wurde demonstriert, dass NiV V und W durch Inhibierung der Transkription von Immungenen

im Stande waren, die Induktion der angeborenen und erworbenen Immunantwort-Kaskade zu unterbinden.

Der zweite Teil dieser Arbeit beschaeftigt sich mit der Frage, ob NiV V oder W Proteine eine regulatorische Aufgabe in der viralen Replikation uebernehmen. Vorherige Berichte hatten dies fuer Nipah Virus selbst (Sleeman *et al.*, 2008) und andere negativ-straengige Viren demonstriert (Atreya *et al.*, 1998; Horikami *et al.*, 1996; Witko *et al.*, 2006). Um die Faehigkeit von V und W Proteinen bezueglich der Regulation der viralen Transkription und/oder Replikation zu bestimmen, wurde von einem bereits existierenden NiV Minireplikon-Assay Gebrauch gemacht (Halpin *et al.*, 2004). In der vorliegenden Studie wurde gezeigt, dass NiV V und W (jedoch nicht C) Proteine die NiV Minireplikon-Aktivitaet in signifikanter Weise reduzierten. Es wurde gezeigt, dass die gemeinsame N-terminale Region die inhibitorische Aktivitaet besitzt. Ko-Immunopraezipitationsexperimente demonstrierten weitehin, dass sowohl NiV V als auch W (jedoch nicht C) im Stande waren, mit NiV N, einer Komponente des NiV Polymerase Komplexes, zu interagieren. Dieses Ergebnis wurde von Immunfluoreszenzexperimenten untermauert, die eine Kolokalisation zwischen NiV N und V und W demonstrierten. Der N-Terminus von NiV V und W, und hierbei speziell die Aminosaeuren 1-50, waren fuer die Bindung von NiV V und W an NiV N verantwortlich. Dies deutete darauf hin, dass V und W durch das Binden des viralen Polymerase Komplexes die virale Replikation inhibiert.

6.3 List of abbreviations

ºC	Degree Celsius
Δ	Delta
µ	micro
aa	Amino acid
AF	Allantoic fluid
APC	Antigen presenting cell
APS	Ammonium persulfate
ATP	Adenosine triphosphate
bp	base pair
BSL	Biosafety level
CAT	Chloramphenicol acetyl transferase
cDNA	copy DNA
CIAP	Calf intestine alkaline phosphatase
CTL	Cytotoxic T cells
Da	Dalton
DC	Dendritic cell
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleic triphosphate
ds	double-stranded
E. Coli	Escherichia Coli
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assy
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
FCS	Fetal calf serum
Fig.	Figure
g	gram
GFP	Green fluorescent protein
h	hour
HC	Heavy chain
HDV	Hepatitis delta virus
HeV	Hendra virus
HIV	Human immunodeficiency virus
hpi	hours post infection
HRP	Horseradish peroxidase
IB	Immunoblot
IFN	Interferon
IL	Interleukin
IP	Immunoprecipitation
ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
JEV	Japanese encephalitis virus
k	kilo

l	liter
LPS	Lipopolysaccharide
M	Molar
m	milli
MHC	Major histocompatibility complex
min	minute
MOI	Multiplicity of infection
mRNA	messenger RNA
n	nano
NDV	Newcastle disease virus
NES	Nuclear export signal
NiV	Nipah virus
NK	Natural killer
NLR	NOD-like receptor
NLS	Nuclear localization signal
NOD	Nucleotide-binding oligomerization domain
NTR	Nontranslated region
OD	Optic density
ORF	open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pol	Polymerase
poly I:C	polyinosinic polycytidylic acid

qRT-PCR	Quantitative Real-Time PCR
rNDV	recombinant Newcastle disease virus
RD	Repressor domain
RLH	RIG-I-like helicase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein particle
rpm	rounds per minute
RT	Room temperature
SDS	sodium dodecyl sulfate
sec	second
ss	single-stranded
SV	Simian parainfluenza virus
TCR	T cell receptor
TLR	Toll-like receptor
Tris	Tris(hydroxymethyl) aminomethane
U	Enzyme unit
UTR	Untranslated region
vec	vector
vRNA	viral RNA
VSV	Vesicular stomatitis virus
WCL	Whole cell lysate
wt	wildtype

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6.6 Statements

Hiermit erklaere ich ehrenwoertlich, dass ich die Dissertation “Characterization of Nipah virus V and W proteins” selbststaendig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Zudem erklaere ich, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem Pruefungsverfahren vorgelegen hat.

Ich habe frueher ausser den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erworben versucht.

Wuerzburg den,

Carolin Alexandra Guenzel