universität wien

DISSERTATION

INFLUENZA B NS1 TRUNCATION MUTANTS: A LIVE ATTENUATED VACCINE APPROACH

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

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1. Summary

The aim of the thesis is to investigate the potential use of mutant replication deficient influenza B viruses with impaired interferon antagonistic function as live attenuated vaccines.

We generated several influenza B viruses containing either carboxy-terminal truncated NS1 proteins of different length or completely lacking the NS1 ORF (ΔNS1-B) employing reverse genetics on Vero cells. Due to a unique, single amino acid mutation M86V in the influenza B M1 protein viral growth in Vero cells was increased, enabling the rescue of a ΔNS1-B virus growing to titers of 8 logs. All viruses showed restricted growth in human alveolar epithelial cells (A549) and in 6 day old human macrophages. The attenuated phenotype of the viruses was associated with induction of antiviral (IFN-α) and pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) early after infection. All vaccine candidates were replication deficient, did not provoke any clinical symptoms and induced neutralizing antibody response in mice and ferrets. Complete protection against homologous challenge with wild-type virus was accomplished after a single intranasal immunization.

So far, the lack of a ΔNS1-B virus component growing to high titers in cell culture has been limiting the possibility to formulate a trivalent vaccine based on deletion of the major interferon antagonist. Our study closes this gap and paves the way for the clinical evaluation of a seasonal, trivalent, live replication-defective ΔNS1 intranasal influenza vaccine.

2. Acknowledgments

Above all, this work owes a lot to my advisors Thomas Muster and Christian Kittel. I thank Thomas Muster for his guidance and financial support. I thank Christian Kittel for his creative thoughts, for his commitment and most of all for his continuous efforts to push me to finish my thesis. I thank Dmitrij Zamarin for advising me in New York. Furthermore I would like to thank many current or former colleagues at Greenhills Biotechnology: especially Julia Romanova and Andrej Egorov for sharing their expertise with me and for their advice, Daniela Ribarits for technical support and interesting conversations during teatime. I thank for helpful impulses and discussions Sabine Nakowitsch, Brigitte Krenn and Bettina Kiefmann. I want to thank Helena Seper and Johannes Humer for being close friends since the first day of my thesis, for their advice and help.

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Last but not least, I am very grateful to my family. I want to thank my mother Ingrid, my father Franz and my brother Thomas for their unconditional support and understanding.

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4. Introduction

4.1. Influenza virus

4.1.1. Structure

Influenza viruses belong to the virus family of *Orthomyxoviridae*, containing eight single-stranded genes in negative polarity. Three types of influenza virus (A, B and C) occur in nature, whereas only influenza virus A and B undergo antigenic drift (A and B) and antigenic shift (only A), defining the epidemiologic and pandemic nature of these viruses and causing significant morbidity and mortality in humans. In contrast to influenza A virus and influenza B virus, as recently discovered, influenza C virus lacks an animal reservoir and is therefore not of major interest concerning outbreaks in humans (Lamb, 1989, Osterhaus et al., 2000, Palese, 2007).

All influenza viruses' posses a common structure (Fig.1). The virus is surrounded by a host derived lipid membrane in which the two viral surface glycoproteins haemaglutinin (HA) and neuraminidase (NA), and the ion channel protein (M2) are embedded. The matrix protein 1 (M1) underlies the lipid envelope forming a protein layer. In the core, each RNA segment is encapsulated by the nucleoprotein (NP) and the three viral encoded polymerase proteins (PB1, PB2 and PA) forming the ribonucleoprotein complex (RNP complex). These RNA segments of influenza A and B possess highly conserved 5' and 3' complementary non coding regions (NCR) of 16 & 15nt in length for influenza A and 10 & 10nt for influenza B viruses, respectively. The NCR are hybridizing with each other, forming a "panhandle" structure (Baudin et al., 1994, Flick & Hobom, 1999). At the same time they are recognized by the viral polymerase complex serving as a promoter controlling the transcription of vRNA. The RNA genome of Influenza A and B virus consists of eight genes coding for 11 proteins due to different open reading frames (ORF) (Compans RW, 1974). Whereas influenza A virus is coding for an additional protein termed PB1-F2, influenza B virus differs from A in expressing an NB protein from the NA ORF (Table 1) (Betakova et al.,

1996, Chen et al., 2001, Imai et al., 2004, Imai et al., 2003). The nuclear export protein (NEP/NS2), formerly believed to be non structural, is also located in the virus particle in a very low copy number (Lamb & Choppin, 1979, Richardson & Akkina, 1991). The small non structural protein 1 (NS1) is only expressed in infected cells and is not being packaged into the virion.

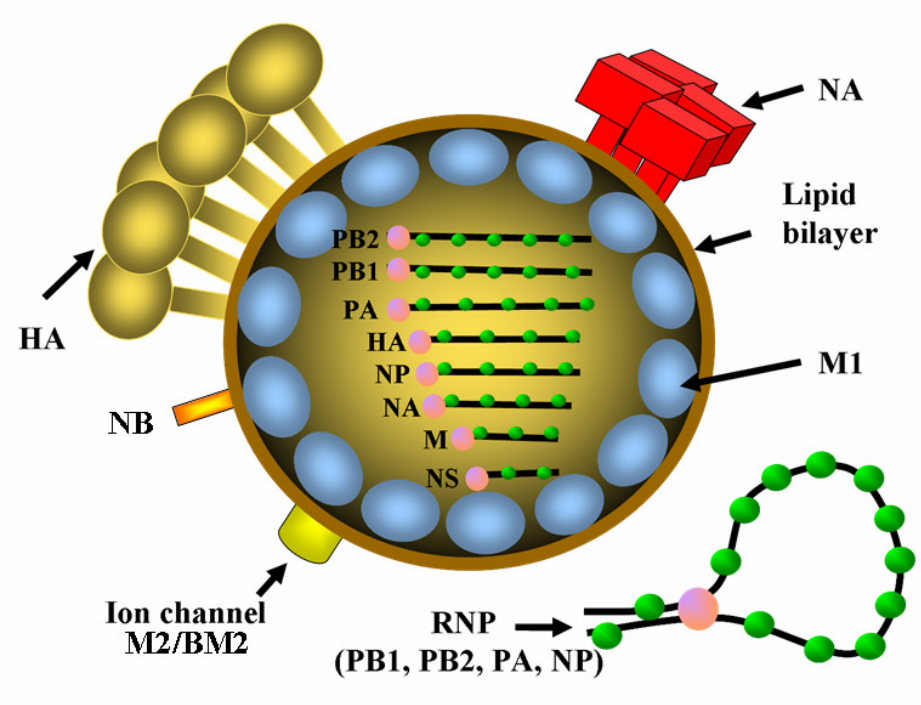

Fig. 1: Schematic drawing of the influenza virus structure

Influenza B viruses are mostly indistinguishable from the A viruses by electron microscopy. In contrast to influenza A virus they have four viral proteins inserted in their lipid membrane, the HA, NA, NB and BM2, whereas the M1 and the RNP complexes make up the interior of the particle.

Table 1: Illustration of gene segments of Influenza A and B (Lamb, 2001)

	A/PR8/34		B/Lee/40		
Seg.	vRNA (nt)	Coding protein (aa)	vRNA (nt)	Coding protein (aa)	Function
1	2341	**PB2** (759)	2396	**PB2** (769)	component of RNA polymerase, cap recognition
2	2341	**PB1** (757)	2368	**PB1** (752)	component of RNA polymerase, endonuclease activity, elongation
		PB1-F2 (87)			pro-apoptotic property
3	2233	**PA** (716)	2304	**PA** (726)	component of RNA polymerase, protease
4	1778	**HA** (566)	1882	**HA** (584)	surface glycoprotein, receptor binding, fusion activity, major antigen
5	1565	**NP** (498)	1841	**NP** (560)	RNA binding, RNA synthesis, RNA nuclear import
6	1413	**NA** (454)	1557	**NA** (466)	surface glycoprotein, neuraminidase activiy
				NB (100)	membran protein, ion channel activity?
7	1027	**M1** (252)	1191	**M1** (248)	matrix protein, interaction with vRNPs and surface glycoproteins, nuclear export, budding
		M2 (97)		**BM2** (109)	membrane protein, ion channel activity, assembly
8	890	**NS1** (230)	1096	**NS1** (281)	multi-functional protein, viral IFN antagonist
		NS2/NEP (121)		**NS2/NEP** (122)	nuclear export of vRNPs

4.1.2. Replication

Influenza virus binds to N-acetyl-neuraminic acid (sialic acid) containing receptors on the host cell surface to initiate viral infection and replication via it's HA protein as illustrated in Fig. 3 (Palese, 2007). Upon adsorption to the receptor the viral particle enters the cell by receptor mediated endocytosis and is internalized into endosomes. Due to the acidic environment in the endosomes, the HA protein undergoes a conformational change, leading to fusion of the viral and cellular membrane (Stegmann, 2000). The viral compartment also gets acidified by M2 protein acting as an ion channel resulting in uncoating (Pinto et al., 1992). This uncoating event leads to the dissociation of the M1 protein from the RNP complex and successively the release of the viral genome (in form of vRNPs) into the cytoplasm. The vRNPs, mediated by the nuclear localisation sequences (NLS) of the NP and polymerase proteins, are then imported into the nucleus for replication and transcription by the virus' own RNA-dependent-RNA-polymerase. Replication occurs later in

infection where vRNA is also transcribed into full length cRNA copies from which more copies of vRNA are made to be later on packaged into new virions. During transcription the subunit of PB2 recognizes and binds m7Gpppm-CAP structures of newly synthesized cellular mRNA. "CAP – snatching" occurs, where the endonuclease activity of PB1 cuts off the CAP and attaches it to the 3' end of vRNA initiating transcription via the 3' OH end of the CAP structure. The viral mRNA acquires the missing polyadenylation tail by the PB1 subunit which elongates the nucleotides until a stretch of five to seven uridines at the 5' end of the vRNA. The RNA polymerase stutters at this uridine stretch and thereby adding the poly (A) tail. Positive sense viral mRNAs are transported out of the nucleus into the cytoplasm for protein synthesis by the ribosomes. After translation the membrane proteins (HA, NA and M2) are transported through the rough endoplasmatic reticulum (rER) and the golgi apparatus to the cellular membrane. During transport different post translational modifications like N-glycolsylation or palmitoylisation occur. The neuraminic acid residues of NA are deleted in order to prevent premature binding to HA.

Due to their NLS the other remaining proteins (PB2, PB1, PA, NP, M1, NS1 and NEP) are imported back into the nucleus to assist in viral replication and vRNP assembly. During late stages of infection, newly synthesized vRNPs are transported to the cytoplasm due to the nuclear export protein (NEP) interacting with the cellular nuclear export machinery. The BM2 protein of influenza B virus is synthesized in the late phase of infection and then incorporated into the virions as a subviral component and plays a critical role in production of infectious virus (Imai et al., 2008, Odagiri et al., 1999). Finally, progeny viruses assemble at the apical surface of the cell and lipid rafts and bud from the host cell plasma membrane.

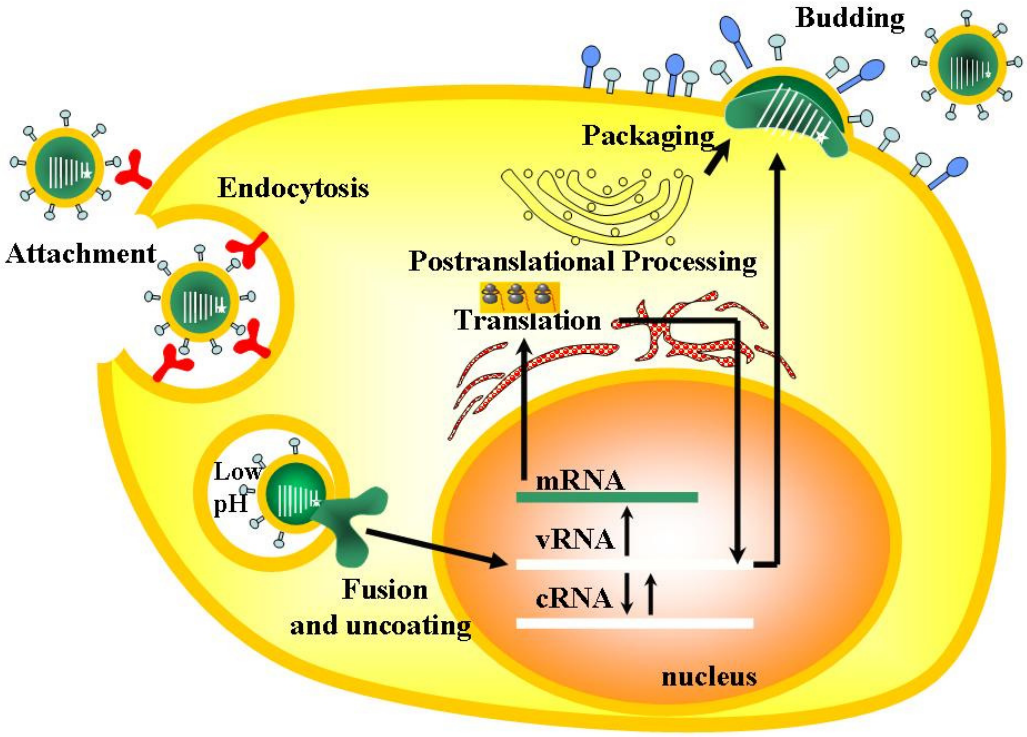

Fig. 2: Influenza virus replication cycle

4.1.3. Structure and function of the NS1 protein

The eleventh gene of the influenza virus, the NS1 protein, is a non structural protein, which is synthesized in the infected cell but is not incorporated into the progeny virus. NS1 is a dimeric, multifunctional protein expressed by all influenza strains. In the case of influenza A virus NS1-A contains 890nt and NS1-B 1096nt coding for two mRNAs (Lamb & Choppin, 1979, Lamb et al., 1980). The first one encoding the NS1 protein of 230 amino acids (aa) or 281aa in length for influenza B viruses and the other mRNA derived by splicing of the NS1 mRNA, encoding the nuclear export protein (NEP/NS2) of 121aa in length for influenza A or 122aa for influenza B viruses, respectively. NEP plays a major role in exporting the newly synthesized vRNPs out of the nucleus. Unspliced NS1 mRNA is exported very efficiently out of the nucleus, ensuring presence of NS1 protein already at early stages of infection and thereby counteracting the onset of the host's antiviral immune response and ensuring prolongation of the viral replication cycle (Alonso-Caplen et al., 1992).

NS1 is a 26kDa protein involved in both protein-RNA as well as protein-protein interactions. Each dimer consists of an N-terminal RNA-binding domain (RBD) and a C-terminal effector domain (ED) as shown in Fig. 3 (Palese, 2007). Due to its NLS in each of the two domains NS1 protein localizes mainly in the nucleus (Greenspan et al., 1988). In the cytoplasm the NS1 protein is found as a result of the NES (nuclear export signal) in the ED (Li et al., 1998). Although there is only 20% sequence homology between NS1-A and NS1-B protein, the function to counteract the hosts own immune system and modulate and regulate the immune response at several stages seems to be alike.

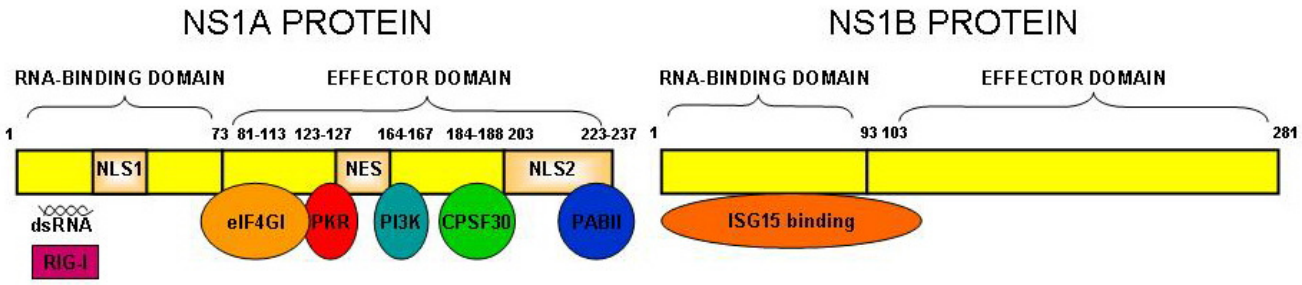

Fig. 3: Binding sites of cellular proteins on NS1 domains of Influenza A and B viruses

One of the functions of the RBD domain of NS1-A is to prevent activation of the cellular Ser/Thr protein kinase R (PKR) (Bergmann et al., 2000, Hatada et al., 1999, Krug et al., 2003). The RBD of NS1-A sequesters dsRNA present in the nucleus away from PKR and/or directly binds to PKR by its ED and therefore prevents its autophosphorylation and activation (Li et al., 2006, Min et al., 2007). Activated PKR induces phosphorylation of its substrate the eucaryontic translation-inhibition factor (eIF-2α) leading to a blockage in cellular protein synthesis which would also affect viral protein synthesis in infected cells leading to inhibition of viral replication (Garcia et al., 2006). The RBD of NS1-B is also able to bind dsRNA and suffices to inhibit PKR activation as demonstrated *in vitro (Dauber et al., 2006)*.

Furthermore, it was shown that the function of the RBD is not only to sequester dsRNA, a putative by-product of viral replication, away from PKR but also from the cytoplasmic antiviral protein 2'-5' oligoadenylate

synthetase (2’5’-OAS) an enzyme binding in its activated conformation to RNase L. Successive activation of RNase L leads to cleavage of both mRNA and rRNA in the cytoplasm inducing a stop in protein expression and hence inhibits viral replication (Silverman, 2007). Whether 2’5’-OAS is activated directly by interaction with the RBD or if dsRNA is sequestrated to prevent activation of this enzyme remains unclear.

Another function of the RBD domain of NS1-A is binding to sequences in the 5’ untranslated regions (UTR) of viral mRNA and interacting with a number of cellular proteins. Thereby recruiting viral mRNA closer to the translation machinery to enhance selective translation of viral mRNAs over cellular mRNAs (Burgui et al., 2003). Thus to increase viral synthesis NS1 was shown to bind to 5’ UTRs, poly(A) binding protein 1 (PABP1), eIF4GI, hStaufen to form multi-protein translation-initiation complexes (Aragon et al., 2000, Burgui et al., 2003). If the same is true for NS1-B has not yet been elucidated.

NS1-B is able to inhibit conjugation of interferon stimulated gene 15 (ISG15) to its downstream binding partners independently form RNA-binding. ISG15 is an ubiquitin-like protein whose expression is transcriptionally induced by IFN-α/β (Lenschow et al., 2007, Yuan et al., 2002, Yuan & Krug, 2001). ISG15 conjugation selectively induces the onset of other early interferon response genes in infected cells, a function which is not shared with NS1-A protein (Krug et al., 2003, Sadler & Williams, 2008).

A function of the NS1-A ED is binding to the subunit of the cleavage and polyadenylation specificity factor (CPSF) and poly(A)-binding protein II (PABII) thereby inhibiting host pre-mRNA processing. CPSF is a cellular protein required for pre-mRNA maturation, in detail for cleavage and polyadenylation (Chen & Krug, 2000). Upon binding of PABII to cleaved mRNA and by its elongation of the poly(A) tail, CPSF joins and promotes further elongation of the poly(A) ensuring correct 3’end processing. The functional processed cellular mRNA can now be exported from the nucleus

(Chen & Krug, 2000). The interaction of the NS1-A ED together with binding to the poly(A) tails of cellular mRNAs and to NS1-I and NS1-BP leads to the selective inhibition of nuclear export of cellular pre mRNAs and the inhibition of splicing, respectively . NS1-B does not interfere with the mRNA processing and is not able to inhibit export of cellular mRNA (Wang & Krug, 1996).

Interestingly, NS1-A is not only involved in suppressing the signaling events mentioned above but is also involved in promoting the phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway (Ehrhardt et al., 2007b, Hale et al., 2006, Hale & Randall, 2007, Shin et al., 2007). At late stages during infection NS1-A ED binds and activates PI3K. Regulation of this pathway induces many cellular changes that maintain cell survival during influenza virus replication, including anti-apoptosis, cell growth, proliferation and cytokine production or signalling. This is again a function that is unique for influenza A and is not shared by influenza B (Ehrhardt et al., 2007a).

The interferon antagonistic function of NS1 was discovered first by the construction of an NS1 deletion virus, lacking the complete NS1 ORF (delNS1) (Egorov et al., 1998, Garcia-Sastre et al., 1998). It was discovered that this virus was severely attenuated in IFN competent systems such as MDCK cells and 11-day old embryonated eggs, but could replicate in IFN deficient systems such as Vero cells and was pathogenic in STAT1-/- mice (Garcia-Sastre et al., 1998). Recombinant influenza viruses with long C-terminal truncations in the NS1 gene also grew well in Vero cells, but were attenuated in growth according to the length of their deletions in MDCK and wild-type mice. This again demonstrates that NS1 is dispensable in the absence of an immune response and is a viral accessory protein not required for viral replication (Egorov et al., 1998, Garcia-Sastre et al., 1998).

4.1.4. NS1 combating the innate immune system

Various hosts counteract influenza invasion by inducing an antiviral response activated by their innate immune system early after infection (Hale et al., 2008). This response involves three major steps:

1. Detection of viral infection and type I interferon (IFN) secretion by the infected cell
2. Binding of IFNα/β to its extracellulary expressed receptors and transcriptional induction of IFN-stimulated genes
3. Induction of an antiviral state of infected and neighbouring cells.

The host cell recognizes influenza infection through a variety of cellular sensors which all trigger signalling events designed to eliminate the invading virus and activate the interferon system. The endosomal Toll-like receptors (TLRs) 3, TLR7 and TLR8 recognize single-stranded RNA and double-stranded RNA, believed to be an intermediate product during viral replication. The cytosolic RNA helicase retinoic acid-inducible gene I (RIG-I) gets activated upon recognition of cytoplasmic influenza-virus-derived ssRNA bearing 5’ triphosphates. Both endosomal and cytosolic sensors start a signalling cascade leading to the transcription of the IFNα/β promoter in the nucleus (Fernandez-Sesma, 2007).

The pattern recognition receptors TLR activate the adaptor molecules MyD88 and lead to further signalling over Toll/IL-R domain-containing adaptor-inducing IFN factor (TRIF) and finally to activation of the IκB kinase-ε (IKKε) and subsequently the interferon response factors (IRFs) and induction of a proinflammatory response (Aderem & Ulevitch, 2000).

The cytosolic RIG-I contains three functional domains, the caspase-recruiting domain (CARD)-like domain responsible for downstream signalling, the helicase domain binding to 5’-triphosphate RNA and finally the repressor domain regulating the activation of RIG-I and only allowing subsequent association with downstream adaptors in the presence of an adequate stimulus like viral RNA (Hornung et al., 2006, Opitz et al., 2007, Pichlmair et al., 2006, Yoneyama et al., 2004). RIG-I is able to discriminate between foreign and endogenous ssRNA via the exposed 5’-3p as during influenza virus replication (Pichlmair et al., 2006). Cellular

mRNA is masked by a 7-methyl-guanosine cap and therefore not recognized by RIG-I. Although the vRNA genome of influenza virus is covered by the NP protein and bound by the vRNP complex, its triphosphate RNA at the 5' end is detected by RIG-I helicase domain and causes a conformational change leading to its activation (Yoneyama et al., 2004). The dogma that dsRNA is the main marker of infection which is common to all viruses and that this dsRNA is the reason for triggering the signalling events leading to an antiviral response is challenged by the ability of RIG-I to recognize specific residues independent of single or double strandedness. Once RIG-I is activated it interacts with a homologous CARD-like domain of the adaptor molecule MAVS (also known as IPS-1, VISA or Cardif) triggering a signalling cascade resulting in the activation of the kinases Traf family-member-associated NF-κB activator – binding kinase 1 (TBK-1) and IκB kinase-ε (IKKε) and thereupon C-terminal phosphorylation of the serine residues of the interferon regulation factor 3 (IRF-3) and IRF-7 (Fitzgerald et al., 2003, Kawai et al., 2005, McWhirter et al., 2004, Meylan et al., 2005, Seth et al., 2005, Sharma et al., 2003, Xu et al., 2005). This provokes a conformational change leading to the translocation and accumulation of activated IRF to the nucleus. Once IRF is present in the nucleus, it associates with the nuclear co-activator CBP/p300, the nuclear factor κB (NFκB) and ATF2/c-Jun to induce transcription of the IFNα/β genes (Fig.6) (Du & Maniatis, 1992, Juang et al., 1998, Lin et al., 1998, Peters et al., 2002, Sato et al., 2000, Wathelet et al., 1998, Weaver et al., 1998).

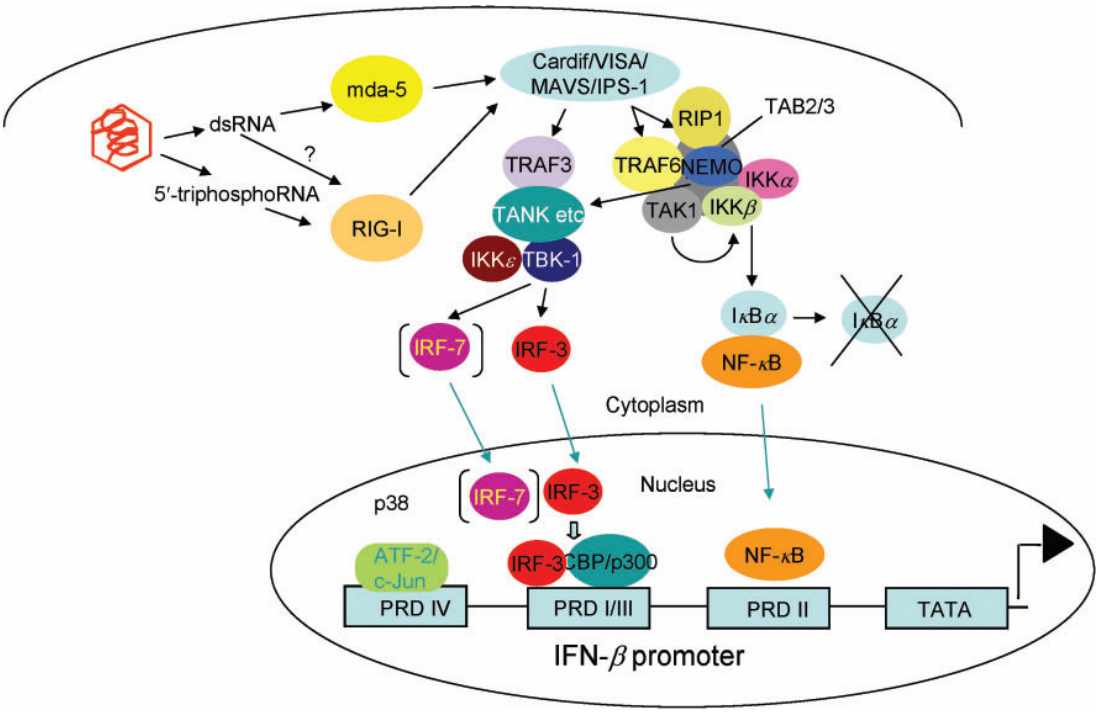

Fig.6. Activation of RIG-I by viral RNA (Randall & Goodbourn, 2008).

NS1 protein of influenza virus has not only the ability to directly interact and bind RIG-I and thus prevent RIG-I mediated production of IFNα/β mRNA, but also the ability to prevent export of mature IFN mRNA from the nucleus by inhibiting posttranscriptional processing of cellular mRNA (Mibayashi et al., 2007, Pichlmair et al., 2006). This is achieved as mentioned before by binding of NS1 to CPSF and PABII. Furthermore it could be shown that the NS1-A and NS1-B protein inhibit phosphorylation and nuclear translocation of IRF-3 (Talon et al., 2000a). This strategy by influenza virus ensures the prevention of cellular production of antiviral proteins and thus allowing continuous viral replication.

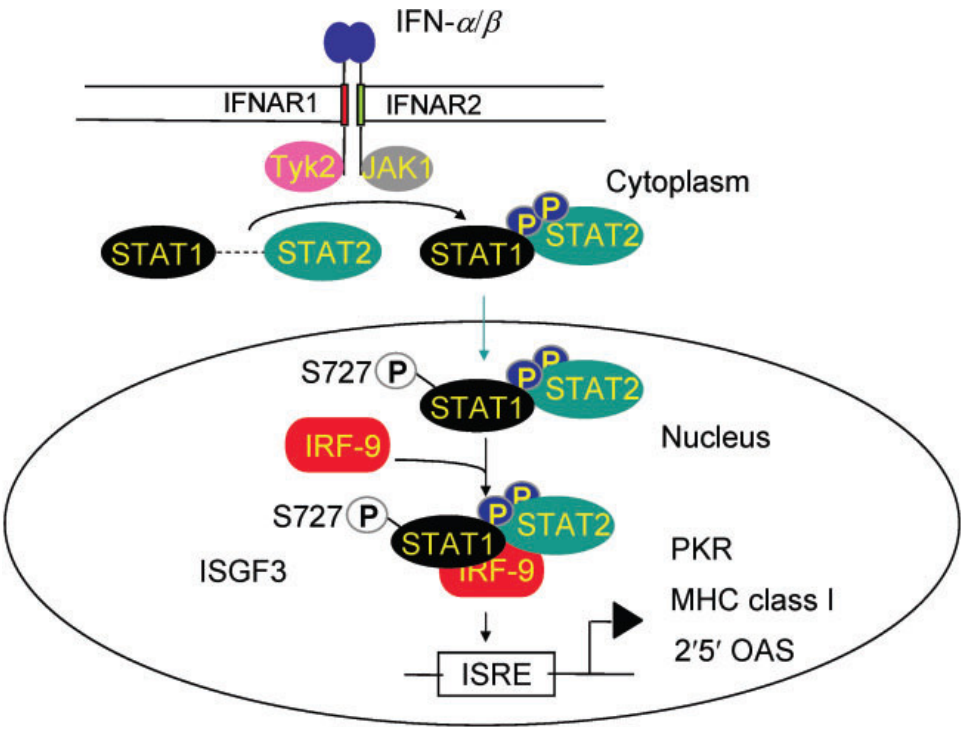

Fig.7. Signalling pathway activated by IFN-α/β (Randall & Goodbourn, 2008).

The produced IFNα/β proteins are secreted from the infected cell and bind to the IFN receptor on the cell surface establishing an antiviral state in the neighbouring uninfected cell and thus preventing viral spread (Fig.7). Upon binding of IFN to the receptor, two Janus protein tyrosine kinases (Jaks), namely Jak1 and Tyk1 get oligomerized and cross activated. They get autophosphorylated and lead to further phosporylation of the signal transducers and activators of transcription (STAT) family. STAT1 and STAT2 form the interferon stimulated gene factor 3 (ISGF-3) transcription complex, which translocates to the nucleus and mediates activation of the transcription of all ISRE controlled genes (Randall & Goodbourn, 2008). The interferon stimulated response element is responsible for the expression of more than 300 interferon-stimulated genes, encoding proteins that have antiviral, antiproliferative or immunomodulatory activities. The antiviral actions are mediated by several ISG proteins like MxA protein, ISG-15, -54, -56, 2’5’OAS and PKR (Decker et al., 2002, Garcia-Sastre & Biron, 2006, Holzinger et al., 2007, Samuel, 2001). Mx proteins belong to the family of dynamin like guanosine triphosphates (GTPases). The Mx proteins were originally identified as factors conferring resistance to lethal influenza A infection in mice. The human MxA accumulates in the cytoplasm and inhibits viral replication. It was shown

that MxA is selectively induced by type I IFNs but not directly by virus infection (Holzinger et al., 2007). The antiviral mechanism against influenza virus infection remains yet to be defined. However, with other RNA viruses, MxA forms complexes with the nucleocapsids of the viruses which are then degraded. As described previously, PKR detects foreign dsRNA in the cytoplasm, leads to inhibition of eIF-2α associated with blocking the translation initiation, whereas 2’5’-OAS, also detecting foreign dsRNA in the cytoplasm, activates RNase L leading to degradation of mRNA (Levy & Garcia-Sastre, 2001). NS1 is interfering with both ISGs, in the case of PKR it is directly binding whereas in the case of 2’5’-OAS, NS1 is sequestering dsRNA in the nucleus. For MxA there has been no evidence of interaction with NS1.

The establishment of an antiviral state is associated with the secretion of such pro-inflammatory cytokines and chemokines as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), IL18, CCL3, CCL4, CCL5 (RANTES), IFN-γ and many more, thereby linking the innate to the adaptive immune response (Julkunen et al., 2001, Kaufmann et al., 2001, Matikainen et al., 2000).

4.1.5. NS1 circumventing the adaptive immune system

After naturally occurring influenza virus infection, the development of a cellular and humoral immune response takes place. Sentinel dendritic cells (DCs) detect an influenza virus infection and upon stimulation start to mature, release proinflammatory cytokines and chemokines and migrate to the lymph nodes. The antigen is then presented to cytotoxic and helper T-cells initiating the adaptive immune response (Hale et al., 2008). Clearance of infection is mediated by cellular immunity involving a T-helper 1 (Th1) response. The optimal Th1 immunity consists of virus specific IFN-γ-secreting CD4 and CD8 T cells recruited to the site of infection that lyse infected cells. Cytokines and chemokines as a part of innate immunity favor the development of antiviral and Th1 type immune responses affecting the adaptive immune response. The NS1 protein of

influenza virus was shown to prevent the induction of several genes involved in DC maturation and migration (Fernandez-Sesma et al., 2006).

Protection against re-infection relies on neutralizing antibodies mainly against the glycoproteins HA and NA. Influenza virus developed mechanisms to avoid recognition by preexisting neutralizing antibodies, a process termed antigenic shift and antigenic drift giving rise to new epidemics and pandemics in humans. Antigenic drift is the accumulation of changes in the antigenic sites of the HA and NA gene due to the high mutation rate of RNA viruses. Additionally, antigenic shift is the capture of novel HA or NA genes by exchange of genes with another virus during reassortment (Palese, 2007).

4.2. Reverse genetics for influenza viruses

Orthomyxoviruses are negative strand RNA viruses rendering their genome un-infectious and resulting in no formation of viral replication when introduced into cells. This is in contrast to positive strand RNA viruses where the RNA resembles mRNA and is itself already infectious.

4.2.1. RNP-transfection

In the case of influenza viruses RNP complexes represent the smallest infectious entity required for encapsidation, transcription and replication of the viral genome, therefore initial experiments focused on reconstituting a functional RNP complex *in vitro* giving rise to the genetic engineering of the influenza virus genome.
In these RNP transfection experiments cDNA for a specific viral segment was synthesized and mixed with purified NP and polymerase complex proteins which were eventually transfected into the cell and super-infected with a helper virus to provide the remaining viral segments (Luytjes et al., 1989). During replication of the helper virus the transfected RNPs were replicated and assembled into new virions. In order to distinguish between helper virus and novel rescued virus a selection pressure has to be applied displaying a huge disadvantage for this system.

4.2.2. Plasmid-only transfection

The generation of influenza viruses entirely from cloned cDNA a decade after the first reverse genetic approach, demonstrated a powerful alternative to the cumbersome limitation of early helper virus infection experiments (Fodor et al., 1999, Hoffmann et al., 2002, Neumann et al., 1999). Each of the eight viral segments was cloned in negative orientation in plasmids containing a human RNA polymerase I promoter on the one side and the hepatitis delta virus ribozyme on the other end in order to ensure that RNA processing gave the correct 3’ end of the vRNA (pPolI

plasmid). Transfection of the eight pPolI plasmids together with the four RNA polymerase II-driven plasmids (pPolII) encoding the NP and the three viral polymerases (PB1, PB2 and PA) into Vero (African green monkey kidney) cells or 293T cells resulted in recovery of infectious influenza virus. Later on, co-cultures of the human 293T cells, which can be efficiently transfected due to possession of the human RNA polymerase I, and the canine MDCK (Madin–Darby canine kidney) cells, which support high growth of influenza virus, were transfected.

Further improvements simplifying the genetic engineering of influenza virus involved the simultaneous transcription of vRNA and mRNA from one plasmid requiring the transfection of only 8 plasmids (Fig.8.). The bidirectional plasmids contain two promoter and termination sites, the viral cDNA is flanked by a human RNA-polymerase I promoter and murine RNA polymerase I terminator as well as a RNA polymerase II promoter and polyadenylation signal. After transfection the cellular RNA polymerase I is responsible for generating vRNA from the plasmid cDNA and transcription by the cellular RNA polymerase II resulted in an mRNA transcript leading to translation by the cellular ribozymes and accumulation of viral proteins. Successively the viral proteins and vRNAs were assembled in the transfected cell and the virus budds from the cell membrane (Hoffmann et al., 2000).

Since cell lines appropriate for human vaccine production (Vero cells) could so far not be transfected with high efficiencies thereby limiting their use in reverse genetics systems. To address this limitation, a reverse genetic system was established reducing the number of plasmids required for virus production (Neumann et al., 2005). The eight pPOLI transcription plasmids for viral RNA synthesis are combined on one plasmid and similarly the polII transcription cassettes for the polymerase complex were combined. By this the number of the required plasmids was significantly reduced and influenza virus could be produced more efficiently in Vero cells than with the previous 12 or 8 plasmid system.

These advances in genetic engineering of influenza virus benefited a better understanding of the structure or function of different influenza virus proteins. Due to the achievements in reverse genetics it is possible

to study the impact of mutations introduced into the cDNA of specific genes, to examine the interaction of viral and cellular proteins or the impact they have on pathogenicity. Plasmid only rescue systems have been described for all three influenza viruses (A, B and C). Furthermore chimeric viruses were rescued in an influenza A background expressing HA and NA proteins from influenza B virus demonstrating that the HA and NA on influenza A can be functionally replaced with the glycoproteins from influenza B (Flandorfer et al., 2003). Reverse genetics experiments also helped to understand the extraordinary virulence of the extinct 1918 pandemic influenza A virus by cloning all 8 genes and subsequent rescue of this virus (Tumpey et al., 2005).

Hereafter, reverse genetics will be a powerful and fast tool to design novel and improved influenza live attenuated vaccine candidates for epidemic and pandemic use.

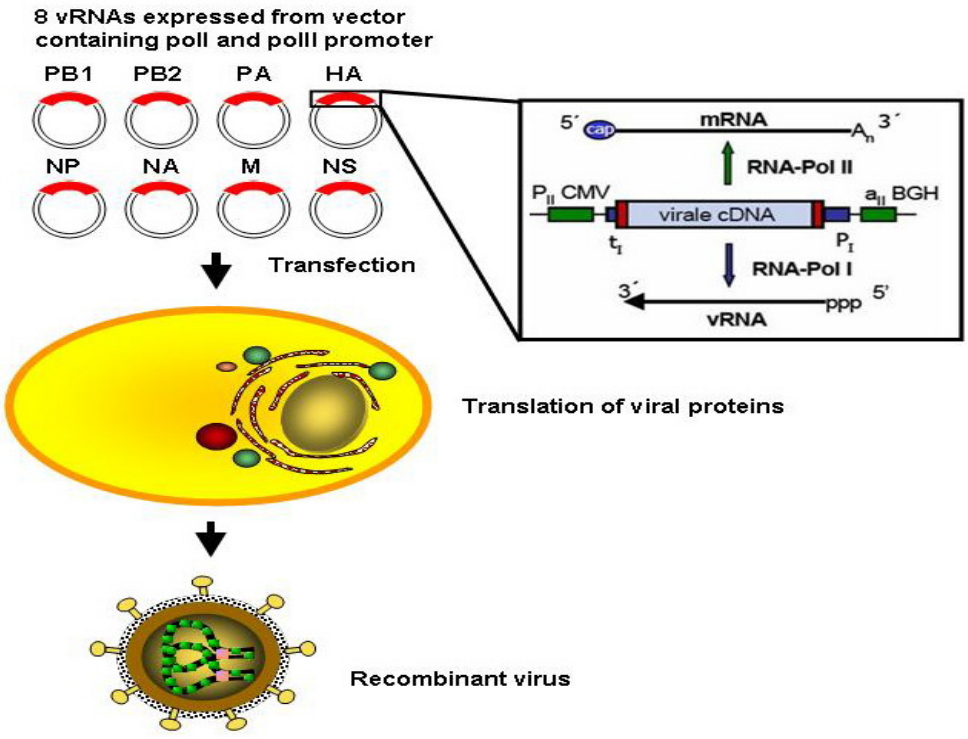

Fig. 8: Reverse Genetics system after (Hoffmann et al., 2002) adapted partly from B. Dauber

RNA-Pol I: RNA-polymerase I; RNA-Pol II: RNA-polymerase II; P_I: humane RNA-Pol I-promoter; t_I: murine terminator; P_{II}CMV: cytomegaly virus RNA-Pol II-promotor; a_{II}BGH: polyadenylation signal of the bovine growth hormone gene

4.3. Vaccine development

As viral growth in embryonated hens' eggs was discovered in the 1930ies, the US military developed the first approved inactivated vaccine for influenza virus in the 1940s and since then a lot has been done to increase safety and efficacy (Hilleman, 2002). To date there are two different approaches licensed, the trivalent inactivated vaccine (TIV) and the trivalent live attenuated influenza virus vaccine (LAIV) (Fig.8). Despite the efficacy of both there is still a need for further development. Not only is the price per dose, the speed and ease of production limiting, but also the cross-protection against variant strains or the choice of the substrate to grow the virus in and the efficacy in immunologically naïve or compromised populations remain critical issues.

4.3.1. Inactivated vaccine (TIV)

There are three types of inactivated vaccines: whole virus, split-product or subvirion and surface-antigen. Nearly all inactivated vaccines are split-product (produced form highly purified influenza virus) or surface antigen vaccines (containing only purified HA and NA glycoproteins). Whole virus vaccine was shown to elicit adverse reactions, especially in children. Due to antigenic drift of the virus, caused by point mutations in the surface antigens, the vaccine formulation is changed almost annually. However, it always consists of three components, influenza A H1N1, H3N2 and influenza B. For preparation of the TIV, the viral reassortants are propagated in embryonated chicken eggs, subsequently UV-inactivated, β-propiolacton or formaldehyde-inactivated and after purification chemically disrupted. Not only can residual amounts of egg proteins remain in the formulation causing problems for allergic populations, but most of all this process is time-consuming, cost-intensive and relies on the availability and supply of SPF (specific pathogen-free) embryonated chicken eggs which could cause problems during a pandemic (Palese, 2006).
The effectiveness of the current TIV depends on the "antigenic match" between novel circulating virus and viral strains used for the vaccination,

the age and the immune status of the vaccinated population. The TIV is injected intramuscularly eliciting a high mean serum IgG antibody response and show a 70-90% efficacy in preventing illness in the healthy population whereas decreased efficacy of only 30-60% in the young and elderly (Cox et al., 2004). However, if efficacy is determined by prevention of death, than 80% of the young and elderly are protected (Nichol et al., 1994, Nichol et al., 1998). Therefore improvements are needed to boost the immune system and to reduce the amount of antigen needed, such as formulation with adjuvants. To date most of the TIVs do not contain an adjuvant. Several have already been licensed like Alum and Mf59 oil-in-water emulsion for conventional influenza vaccines. A huge supply problem would be met, if under adjuvant conditions the dose of antigen currently present per vaccine (45µg of HA) could be significantly reduced and still stimulate an adequate immune response. Researchers are now investigating liposome-like preparations containing cholesterol and viral particles as immuno- stimulating complexes (ISCOM) and could demonstrate their efficacy even by nasal administration in mice (Sambhara et al., 2001). However, no protection was observed in a macaque model against distant influenza drift variants (Rimmelzwaan et al., 2001). Preparations of liposomal-like complexes (INFLUSOME-VAC) admixed with liposomal human Interleukin-2 (IL-2), to stimulate a better cross-reactivity, appear to be both safe and more immunogenic than the currently used vaccine in the elderly (Ben-Yehuda et al., 2003). Another approach is the use of the heat-labile *Escherichia coli* toxin complexed with lecithin vesicles together with TIV for intranasal administration (Glueck, 2001). Further research has to be done in order to make this approach safer, for this specific adjuvant was shown to be associated with Bell's palsy disease. Other attempts include synthetic adjuvants such as synthetic lipid A, muramyl peptide derivates, cationic molecules and poly I:C (Ichinohe et al., 2005). Virosomes consisting only of reconstituted viral envelopes and completely lacking RNA have also been shown to be effective alternate approaches for influenza vaccines (Huckriede et al., 2005).

4.3.2. Live vaccines

4.3.2.1. Live cold-adapted vaccine (LAIV)

LAIVs are administered intranasally displaying a huge advantage over TIV. Cold-adapted influenza vaccine was used in Russia already for decades, was shown to be protective in children and demonstrated no side effects. Furthermore as it is always the concern of live vaccines, no sign of spread or virulent revertants could be found (Wareing et al., 2001). Recently it was also licensed in the US as *Flumist* by MedImmune (Vesikari et al., 2006b). In order to generate attenuated cold-adapted strains the influenza virus isolate was passaged in embryonated eggs or primary chicken kidney cells several times to adapt them to growth at low temperature of 25°C (Herlocher et al., 1993). The resulting master strain is temperature sensitive (*ts*) and cold-adapted (*ca*) bearing several point mutations restricting growth at high temperatures as 37°C. Thereby exhibiting a strong attenuated phenotype in animals as well as humans replicating only in the upper respiratory tract in which temperatures do not exceed 32-33°C. The three NA and HA proteins of the current epidemic strains are semi-annually introduced by genetic reassortment. The resulting 6:2 reassortants contain 6 non-surface proteins of the master strain and the HA and NA from circulating A and B viruses according to the WHO recommendations.

LAIV elicits a local IgA mucosal antibody response in the upper respiratory tract, lower but nevertheless detectable serum IgG antibody titers than TIV and a longer lasting virus specific T cell response suggesting that this vaccine may provide better cross-protection against antigenic drift variants. The protection however is better in certain age groups from 6 months to 9 year old children, nevertheless resulting in an overall efficacy of 73-96% in a population of 6 months to 18 years (Vesikari et al., 2006a). Additional advantages besides the nasal application poses the development of reverse genetics making it possible to generate the 6:2 reassortants much faster than by classical reassortment and furthermore abolish the presence of adventitious agents in the vaccine preparation.

4.3.2.2. Live recombinant vaccine

Since the development of genetic engineering accompanied by its possibility to alter every single gene in the influenza virus, new vaccine approaches can be considered. So far none of them has been yet licensed but attempts and improvements have been made by several groups. It is possible to tailor made strains with specific properties leading to their attenuation.

One attempt was the exchange of promoter regions of the NA protein of influenza A and B viruses, which led to attenuation in mice (Muster et al., 1991). Another group introduced mutations by site-directed mutagenesis into the PB2 gene leading to temperature-sensitive (ts) and attenuated phenotype in mice and ferrets (Murphy et al., 1997, Parkin et al., 1997). Another attenuation mechanism was met by the generation of a virus lacking the nuclear export (NS2/NEP) protein. These virus particles undergo just a single round of replication, expressing the viral proteins but due to the missing NS2/NEP protein no infectious viral particles are formed (Watanabe et al., 2002b). Elimination of the M2 gene renders a virus to grow efficiently in tissue culture but poorly in mice, a prerequisite for a potential live virus vaccine candidate (Watanabe et al., 2002a). Production of such viruses in tissue culture can be achieved by the use of complementing cell lines expressing the missing protein. Additionally virus-like-particles (VLPs) have been shown to generate a mucosal immune response when applied to a mucosal surface especially in combination with an adjuvant. VLPs consist of influenza virus structural proteins like HA, NA, M1 and M2 which are sufficient for assembly and budding from the cell membrane. On this basis it is possible to produce a vaccine in large quantities due to the usage of Sf9 insect cells. Furthermore VLPs demonstrated to be effective in an animal model, however the data of clinical trials are so far not available (Galarza et al., 2005).

4.3.2.3. NS1 live-attenuated vaccine

Finally, one approach comes from the finding that the non-structural protein (NS1) has IFN antagonistic properties (Egorov et al., 1998, Garcia-Sastre et al., 1998). Influenza viruses lacking NS1 are not able to counteract the host immune response and therefore type I IFN production is induced and viral replication is blocked. Truncations in the NS1 protein of influenza A and B viruses were shown to result in an altered phenotype with different virulence characteristics in mice (Hai et al., 2008, Talon et al., 2000b). The level of attenuation directly correlated with the length of the NS1 protein (Baskin et al., 2007, Ferko et al., 2004, Hai et al., 2008, Quinlivan et al., 2005, Richt et al., 2006, Solorzano et al., 2005). Therefore an optimal balance between efficacy and safety has to be met, retaining the virus' replication deficiency and inducing significant amounts of IFN which cannot be overcome by the virus. Due to the fact that viruses with an impaired NS1 protein induce higher levels of IFN than corresponding wild-type viruses, the produced IFN enhances the production of primary antibodies and contributes to the activation of antigen presenting dendritic cells (Stasakova et al., 2005). Thus the immune response to NS1 truncated viruses might be enhanced and demonstrate increased efficacy when compared to conventional TIV or LAIV. Moreover IFN acts as an additional self-adjuvant (Ferko et al., 2004, Le Bon et al., 2001).

These IFN-sensitive vaccine candidates require tissue culture cell lines that are not able to produce and respond to IFN after influenza virus infection. Therefore Vero cells are one cell line of choice, as they do not produce IFN due to a natural deletion of the IFN genes (Desmyter et al., 1968, Mosca & Pitha, 1986) and furthermore they pose the advantage that they are a certified cell line suitable for vaccine production promoting the growth of viruses with an impaired NS1 function equally to wild type. The overall benefits of these live influenza vaccine candidates with impaired NS1 protein over TIV are the replication-deficient phenotype, the intranasally administration, the presumable efficacy in immunologically naïve populations, the triggering of a mucosal immunity, the lack of

adventitious agents and the production in large quantities due to tissue culture production.

4.3.3. DNA vaccination

Another approach very distinguished from TIV and LAIV is DNA vaccination. DNA vaccines consist of *Escherichia coli* derived plasmid DNA encoding one or more influenza proteins of interest. The immune response is only directed against the inserted protein not the vector itself. A DNA vaccine expressing HA protein was shown to be effective in mice, ferrets and chicken when injected intramuscularly or given with a bio ballistic gene gun(Donnelly et al., 1995, Kodihalli et al., 2000, Webster et al., 1994). With this vaccination method, both a humoral and cell-mediated immune response is induced, due to the expression of the antigens *in situ.* The great potential of this technology is the speed of vaccine generation, the ease of production and its low cost. Despite the drawback that experiments so far were limited to animal models, efforts invested into the development of a universal approach inducing protective humoral and cell mediated immunity to a variety of antigens seem worthwhile.

Vaccine	Immune response	Advantages	Disadvantages
Inactivated Influenza Virus (TIV)	AB	In clinics	Adjuvant needed, egg production, IM administration
Live attenuated Influenza virus (LAIV)	T cell + AB	IN administration, in clinics	Revertants bear safety issue, egg production
Virus like particles (VLPs)	AB	Ease of production, tissue culture	No production capability so far
Live-recombinant Influenza virus (ΔNS1)	T cell +AB	Replication-deficient, no revertants, IN administration, tissue culture, self-adjuvanted	
Plasmid DNA	T cell + AB	Easy to develop and produce	Efficacy in humans not yet established

Candidate influenza vaccines

Fig. 8: Table of influenza virus candidates

IM: intra muscular; IN: intranasal; AB: antibody; adapted from Kawaoka et al.

5. Objective of thesis

The aim of the thesis was to develop a reverse genetic system for the generation of contemporary influenza B viruses encoding C-terminally truncated NS1 proteins or completely lacking the ORF of NS1. Rescued viruses were selected for high growth on Vero cells. The efficacy and immunogenicity in animals was demonstrated and their potential for the use as vaccine candidates in a trivalent live-attenuated vaccine formulation was assessed.

The thesis is a collection of results outlined in 2 chapters, a published paper in chapter 6 and a manuscript submitted for publication in chapter 7.

6. Influenza B mutant viruses with truncated NS1 proteins grow efficiently in Vero cells and are immunogenic in mice

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6.1. SUMMARY

We generated contemporary influenza B viral strains encoding carboxy-terminally truncated NS1 proteins. Viable viruses containing the amino-terminal 14, 38, 57 or 80 amino acids of the NS1 protein were rescued on Vero cells. The influenza B NS1 truncation mutants are impaired in their ability to counteract interferon production, induce antiviral pro-inflammatory cytokines early after infection and show attenuated or restricted growth in interferon-competent hosts. In Vero cells all obtained viruses replicate to high titers comparable to the influenza B wild-type virus. Mice, which received a single, intranasal immunization of the NS1 truncation mutants elicit antibody response and protection against wild-type virus challenge. Therefore, these NS1 truncation mutants should prove useful as potential candidates for live attenuated influenza vaccines.

6.2. INTRODUCTION

Influenza viruses belong to the *Orthomyxoviridae* family and are enveloped, negative-sense RNA viruses with a segmented, single-stranded genome (Wright, 2001). Whereas influenza A virus has a broad host reservoir in many avian and mammalian species, influenza B virus is thought to be almost exclusively restricted to humans (Wright, 2001). Most of the proteins expressed by influenza A and B viruses are believed to have similar functions despite the biological and epidemiological differences between these two viruses. Clear exceptions are the pro-apoptotic PB1-F2 protein uniquely found in most influenza A virus strains (Chen et al., 2001) and the NB protein solely expressed by influenza B, which contributes to virulence (Imai et al., 2008, Shaw et al., 1983).

Various hosts counteract viral invasion by inducing an antiviral response activated by their innate immune system. This response involves three major steps: (a) detection of viral infection and type I interferon (IFN) secretion by the infected cell, (b) binding of IFN to its extracellulary expressed receptors and transcriptional induction of IFN-stimulated genes leading to (c) the induction of an "antiviral state" of infected and neighboring cells. In detail, the activation of the host-type I IFN system is mediated predominantly upon recognition of influenza-virus-derived RNA bearing 5' triphosphates by the cytoplasmatic RNA helicases RIG-I (Pichlmair et al., 2006, Yoneyama et al., 2004). Interaction of RIG-I with MAVS (also known as IPS-1, VISA or Cardif) triggers a signaling cascade resulting in the activation of the kinases TBK-1 (Traf family-member-associated NF-κB activator – binding kinase 1), IKKε (IκB kinase-ε) and thereupon phosphorylation of the transcription factor IRF-3 (interferon regulating factor 3) (Fitzgerald et al., 2003, Kawai et al., 2005, McWhirter et al., 2004, Meylan et al., 2005, Seth et al., 2005, Sharma et al., 2003, Xu et al., 2005). Activated IRF-3 translocates and accumulates in the nucleus. Together with the nuclear coactivator CBP/p300, NFκB (nuclear factor κB) and ATF2/c-Jun (activating transcription factor 2) induce transcription of IFN-α/β (Du & Maniatis, 1992, Juang et al., 1998, Lin et al., 1998, Peters et al., 2002, Sato et al., 2000, Wathelet et al., 1998,

Weaver et al., 1998, Yoneyama et al., 1998). IFN-α/β are secreted from the infected cell and bind to the IFN receptor on the cell surface. This triggers a signal cascade through the JAK/STAT pathway leading to the formation of the ISGF-3 (Interferon stimulated gene factor 3) transcription complex, which, in turn is responsible for the expression of more than 100 interferon-stimulated genes, some of them associated with antiviral activity, e.g. Mx proteins, ISG-15, -54, -56, 2',5'OAS (2',5'- oligo (A) synthetase) and PKR (Protein kinase R) (Garcia-Sastre & Biron, 2006, Holzinger et al., 2007, Samuel, 2001). This establishment of an antiviral state is associated with the secretion of such pro-inflammatory cytokines and chemokines as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), IL-18, CCL3, CCL4, CCL5 (RANTES), IFN-γ and many more (Julkunen et al., 2001, Kaufmann et al., 2001, Matikainen et al., 2000).

During its evolution, viruses have developed a mechanism to overcome this defense strategy of the innate immune system. Earlier on, it could be demonstrated that the non-structural protein 1 (A/NS1) plays this role for influenza A viruses by antagonizing the IFN response of the infected cell (Egorov et al., 1998). In detail, A/NS1 has been associated with a number of regulatory functions during virus infection: (a) inhibition of host mRNA polyadenylation and pre-mRNA splicing, which contributes to the virus-induced shutoff of host protein synthesis (Lu et al., 1994, Nemeroff et al., 1998), (b) binding to the poly(A) tails of mRNAs, thus inhibiting their nuclear export (Qiu & Krug, 1994), (c) regulation of viral RNA polymerase activity (Marion et al., 1997, Shimizu et al., 1994), (d) interaction with the subunit of the cleavage and polyadenylation specificity factor (CPSF), poly(A)-binding protein II (PABII) and NS1-BP, leading to the selective inhibition of nuclear export of cellular mRNAs and the inhibition of splicing, respectively (Nemeroff et al., 1998, Wolff et al., 1996, Wolff et al., 1998), (e) blocking the activation of such transcription factors as IRF-3, IRF-7, NFκB and ATF-2/c-Jun thereby inhibiting the induction of IFN-α/β (Levy et al., 2002, Ludwig et al., 2006, Ludwig et al., 2002, Talon et al., 2000a, Wang et al., 2000), (f) sequestration of dsRNA, preventing activation of the IFN cascade and, finally, (g) binding to Mx protein, 2',5'-OAS and PKR, the latter being one of the major antiviral proteins responsible for

inhibiting translational expression of cellular proteins (Dittmann et al., 2008, Lu et al., 1995, Min & Krug, 2006, Samuel, 2001).

Although the influenza B virus NS1 protein (B/NS1) only has about 20% sequence homology with A/NS1, many of the functions are shared by B/NS1 with some distinct exceptions. Differently to influenza A virus, no NS1-mediated nuclear retention of mRNAs could be observed during influenza B infection (Wang & Krug, 1996). Only influenza B/NS1 inhibits conjugation of ISG15 protein to its downstream binding partners and selectively induces the onset of early response genes in such infected cells as ISG56 (Lenschow et al., 2007, Yuan et al., 2002, Yuan & Krug, 2001), a function which is not shared with influenza A viruses (Kim et al., 2002, Talon et al., 2000b, Wang & Krug, 1996). The N-terminal RNA-binding domain (RBD) of influenza B/NS1 suffices to inhibit PKR activation and has IFN antagonist potential (Donelan et al., 2004). In the context of a recombinant virus the C-terminal part of B/NS1 is required to inhibit IFN-α/β induction in epithelial cells (Dauber et al., 2006). Some other functions of NS1 are common to both influenza types, e.g. binding to RNA via an N-terminal RBD.

It was shown that influenza A viruses lacking a functional NS1 protein (ΔNS1) were attenuated in such IFN-competent hosts as MDCK cells and wild-type (wt) mice, but replicated in IFN-deficient Vero cells and STAT1- (signal transducer and activator of transcription 1) or PKR knock-out (ko) mice (Egorov et al., 1998, Garcia-Sastre et al., 1998, Palese et al., 1999). Despite replication deficiency in normal hosts, animals immunized with ΔNS1 viruses elicit a strong immune response demonstrated by increased antibody titers, elevated cytokine expression, activation of T-helper cells and protection against wt virus challenge (Donelan et al., 2003, Ferko et al., 2004, Talon et al., 2000b).

Using a reverse genetic system in Vero cells, we describe the construction and properties of isogenic influenza B mutant viruses encoding carboxy-terminal truncated NS1 proteins differing in length from 80 to 14 amino acids (aa). Analogous to the data found for influenza A NS1 mutants (Egorov et al., 1998), these viruses are attenuated in IFN-competent hosts but replicate to high titers in Vero cells. All constructed mutants are

able to induce antibody immune response upon intranasal (i.n.) immunization of mice and protected animals after homologous influenza virus challenge.

6.3. MATERIALS & METHODS

6.3.1. Cells, viruses and viral infections

Vero cells (ATCC CCL-81) were used for transfection experiments, for propagating viruses and virus titrations. Vero cells were adapted to and cultivated in serum-free OptiPro media (Invitrogen) with 4mM L-glutamin (Gibco). A549 cells (ATCC CCL-185) were cultivated in Dulbecco-modified Eagle medium (DMEM; Gibco) and used for growth curves of rescued viruses. Immortalized PKR knock out (ko) mouse embryonic fibroblasts (MEF) were obtained from H. Unger (University of Veterinary Medicine, Vienna, Austria), cultivated in Minimal Essential Medium (MEM; Gibco) with 10% fetal calf serum (FCS; Gibco) and used for growth curves of rescued viruses. Primary human nasal epithelial cells (HNEC) (Provitro) were cultivated in Airway Epithelial Cells Growth Medium (Provitro). Influenza virus B/Vienna/33/06 (B/Malaysia/2506/04-like) was provided by M. Redlberger (Institute of Virology, Vienna, Austria). Influenza B/Thüringen/02/06 (B/Jiangsu/10/03-like) virus was obtained from B. Schweiger (German National Reference Center for Influenza; Robert Koch Institute, Berlin, Germany). Both viruses were adapted to grow on serum-free Vero cells. For determination of viral replication, indicated cells were washed with phosphate-buffered saline (PBS) and incubated with virus at the indicated multiplicities of infection (MOI) at room temperature for 30 min. Inoculum was removed, serum-free OptiPro media containing 5µg/ml Trypsin (Sigma) and 250µg/ml Amphotericin B (Brystol Meyers) was added and cells were incubated at 33°C and 5% CO_2. After indicated time points, supernatant of infected cells was analyzed for viral titer by $TCID_{50}$ and viral titers were calculated.

For the measurement of IFN and cytokines on HNEC, cells were infected at an MOI of 2. After 30 min of incubation, inoculum was removed, 1ml of fresh culture medium was added and cells were incubated at 33°C and 5% CO_2. Cell culture supernatants were harvested 24h post infection (p.i.) and analyzed for the presence of IFN-β, TNF-α, IL-1β and IL-6.

6.3.2. Generation of NS1-truncated viruses

HA and NA genes of B/Thüringen/02/06 virus and the remaining 6 genes of the B/Vienna/33/06 master strain including non-coding regions were sequenced and each cloned into a phW2006 vector, a synthetically produced vector similar to pHW2000 (Hoffmann et al., 2002). It contains a PolI and PolII promoter and terminator for the bicistronic expression of influenza genes. The NS gene was modified by site-directed mutagenesis (Stratagene) to express an NS1 protein of 14, 38, 57 or 80 aa, respectively. Translation was terminated by two consecutive in-frame stop codons. The cDNA downstream of the stop codon cassette up to the splicing signal of NS2/NEP (position 634nt) was deleted by inverse PCR. A mutation at nt position 280 (A-G) in the M gene resulting in a change at amino acid position 86 (M-V) of the M1 protein was introduced by similar way. Reassortants having HA and NA genes from B/Thüringen/02/06 and all other genes from B/Vienna/33/06 (6:2 composition) were rescued by transfection of Vero cells as described previously (Kittel et al., 2005). This 6:2 constellation was chosen because wt isolates containing all 8 genes from B/Vienna/33/06 lack replication in mice. Viruses were named according to the size of the NS1 protein, i.e. NS1-14, NS1-38, NS1-57, NS1-80 and NS1-wt, respectively. The expected sequences of HA, NA and NS genes of rescued viruses were confirmed by sequencing and for NS additionally by analysis of amplified RT-PCR products. All sequences are available upon request.

6.3.3. Isolation, generation and infection of immature monocyte-derived macrophages

Peripheral blood monocytes (PBMCs) obtained from leukocyte-rich buffy coats of healthy donors were purified by standard gradient centrifugation with Ficol-Paque (GE HealthCare). The CD14-positive cells were separated by immunomagnetic sorting using VARIOMACS technique (Miltenyi BiotecGmbH) according to the manufacturer's procedure. Isolated CD14-cells were cultured in polystyrene 6-well plates with hydrophobic surface (Greiner bio-one). 2 x 10^6 cells per well were cultivated in 2ml RPMI 1640 medium (Invitrogen) containing 10% FCS (Hy Clone) at 37°C in a humidified 5% CO_2 atmosphere in the presence of 250 U/ml recombinant human granulocyte-macrophage colony stimulating factor (Berlex) for 7 days. Every second day, cells were fed with 1ml RPMI-1640 medium containing 10% FCS. At day 7, 1 x 10^6 macrophages were collected and transferred to polystyrene tubes (Falcon), washed with serum-free medium and infected with the viruses described above at an MOI of 2. After incubation for 30 min, cells were spun down and resuspended in 1ml RPMI-1640 medium (Gibco) containing 10% FCS and incubated at 37°C in 5% CO_2. Supernatants were harvested at 24h p.i. and analyzed for the presence of IFN-α, TNF-α, IL-1β and IL-6.

6.3.4. Cytokine measurement in cell-culture supernatants

For cytokine measurement (TNF-α, IL-1β and IL-6) a Luminex 100 system was used (Beadlyte Human Multi-Cytokine Detection System 2) according to the manufacturer's instructions. The amounts of IFN-α/β were determined by quantitative cytokine-specific ELISA kits (PBL Biomedical Laboratories), following manufacturer's instructions and shown as one representative of three independent experiments.

6.3.5. Immunization and challenge of mice

Seven animals per group of 6 to 8-week-old female BALB/c mice were i.n. infected under ether anesthesia with the indicated viruses at 5 x 10^5 $TCID_{50}$/mouse or with serum-free Optipro media (control group). Three days post immunization, 3 animals per group were sacrificed to determine the viral load in lungs and nasal turbinates. For this purpose, a 10% tissue extract in SPGN buffer was prepared by grinding the tissue sample with a rotor homogenizer. The suspension was then centrifuged at 2000 x g for 10 min and supernatants were analyzed for viral yield by $TCID_{50}$/ml. Blood was collected from the murine retro-orbital venous plexus 29 days following priming, the sera were prepared and stored at -20°C. The remaining animals were challenged with influenza NS1 wt strain (5 x 10^5 $TCID_{50}$/mouse) 32 days post immunization and were sacrificed 3 days post challenge to analyze the viral load in their lungs as described above.

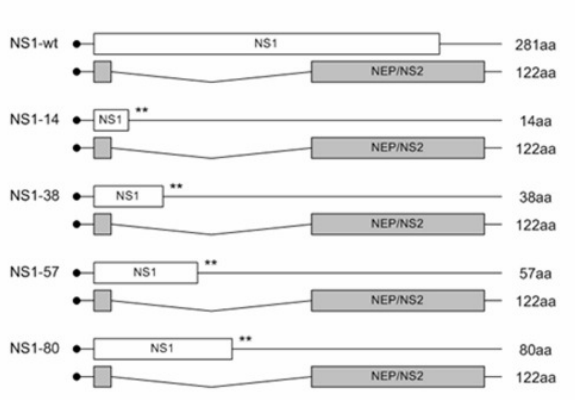
6.3.6. Influenza-specific IgG ELISA

96-well microtiter plates were coated with influenza B/Thüringen/02/06 adjusted to 50 HA units per well in a carbonate buffer (pH 9.6). Coated plates were incubated over night at 4°C, then washed with PBS containing 0.1% Tween20 (PBS/Tween) and blocked with PBS/Tween plus I-block (Applied Biosystems) 5mg/ml (PBS/Tween/I-block). Serial dilutions of sera from immunized and, as a control, from naïve mice in PBS/Tween/I-block were applied to the plates (50µL/well) and incubated for 1.5h at room temperature. After washing, secondary rabbit anti-mouse IgG1 or IgG2a antibodies conjugated with horseradish peroxidase (Invitrogen) were added. After an additional washing step, plates were stained with Ultra-TMB substrate (Thermo). The reaction was stopped with 4M H_2SO_4 and absorbance was measured at a wavelength of 450nm. The cut-off value was defined as the mean value of absorption of blank plus three standard deviations and shown as one representative of two independent experiments.

6.4. RESULTS

Rescue of influenza B mutant viruses encoding C-terminally truncated NS1 proteins in Vero cells. Eight plasmids expressing HA and NA from B/Thüringen/02/06 and the remaining six genes from B/Vienna/33/06 master strain were used to generate influenza B virus NS1 truncation mutants by reverse genetics. We found that this 6:2 gene constellation leads to high rescue efficiency in Vero cells (data not shown). Translation of NS1 was terminated by two consecutive in-frame stop codons at aa positions 14, 38, 57 and 80, respectively. The non-translated part downstream of the stop codons up to the splicing signal of NS2/NEP was deleted to prevent reversion to wt NS1. A schematic representation of the constructs is shown in Fig. 1a. The NS1 truncation mutants were rescued in Vero cells as was the recombinant wt virus. The resulting rescued truncation viruses containing the N-terminal NS1-specific 14, 38, 57 and 80 aa, respectively, were designated NS1-14, NS1-38, NS1-57, NS1-80 and NS1-wt. The different sizes of the NS gene of generated mutant viruses analyzed by RT-PCR confirmed the identity of the specific truncation as shown in Fig. 1b. Despite several attempts, we did not succeed in rescuing a ΔNS1-B virus, in which the ORF of NS1 is completely deleted and NS2/NEP is expressed as monocistronic mRNA.

Fig. 1(A)



(B)

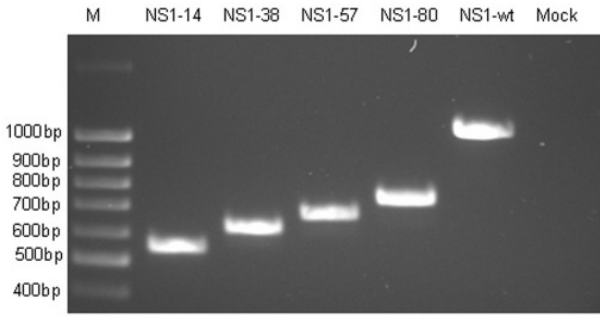


Figure 1: Generation of recombinant wt influenza B virus and NS1 truncation mutants. (A) Schematic representations of the NS genes and NS specific mRNAs of the wt, NS1-14, NS1-38, NS1-57 and NS1-80 truncation viruses. The asterisks (**) indicate two consecutive in frame stop codons. The part downstream of the stop codons up to the splicing signal of NEP/NS2 was deleted. (B) RT-PCR analysis of viral NS segments. RNA was isolated from the wt influenza B virus and from the NS1 truncated viruses and the NS segments were reverse transcribed and amplified by PCR. The resulting products were separated on a 2% agarose gel and stained with ethidium bromide. Sizes are indicated.

Influenza B NS1 truncation mutants replicate efficiently in IFN-deficient Vero cells but are attenuated in A549 cells and MEFs of PKR ko mice. NS1 truncation mutants and wt virus were evaluated for their potential to grow in IFN-competent A549 and IFN-deficient Vero cells (Fig. 2a+b). All NS1 truncation mutants showed similar growth kinetics in Vero cells reaching titers in the range of 10^7 to 10^8 $TCID_{50}$/ml, which are comparable to those found with the wt virus. Replication of NS1-truncated viruses was severely attenuated in IFN-competent A549 cells as compared to Vero cells. While NS1-wt virus replicated to high titers of 4.4 x 10^7 $TCID_{50}$/ml, the growth of NS1-80 virus was significantly impaired with a difference of approximately 4 orders of magnitude. The NS mutants expressing an NS1 protein of less than 80 aa were even more attenuated showing almost complete restriction in growth in A549 cells with titers close to or below the detection limit of 2 x 10^2 $TCID_{50}$/ml. Similar results were observed in human macrophages where only wt virus was able to replicate to 6 logs and all NS mutants did not replicate (data not shown).
In the next step we investigated whether knocking out PKR, an antiviral protein known to be counteracted by the N terminal domain of the influenza B NS1 protein, is sufficient to restore viral growth in interferon competent cells. Therefore, we compared the truncated viruses' ability to grow in mouse embryonic fibroblasts derived from PKR ko mice (Fig. 2c). This cell line supported the growth of wt virus of more than 7 logs. The NS1-80 virus replicated almost to wt levels whereas NS truncation mutants expressing a NS1 protein of less than 80 aa showed reduced growth properties by approximately 3 logs.

Fig.2

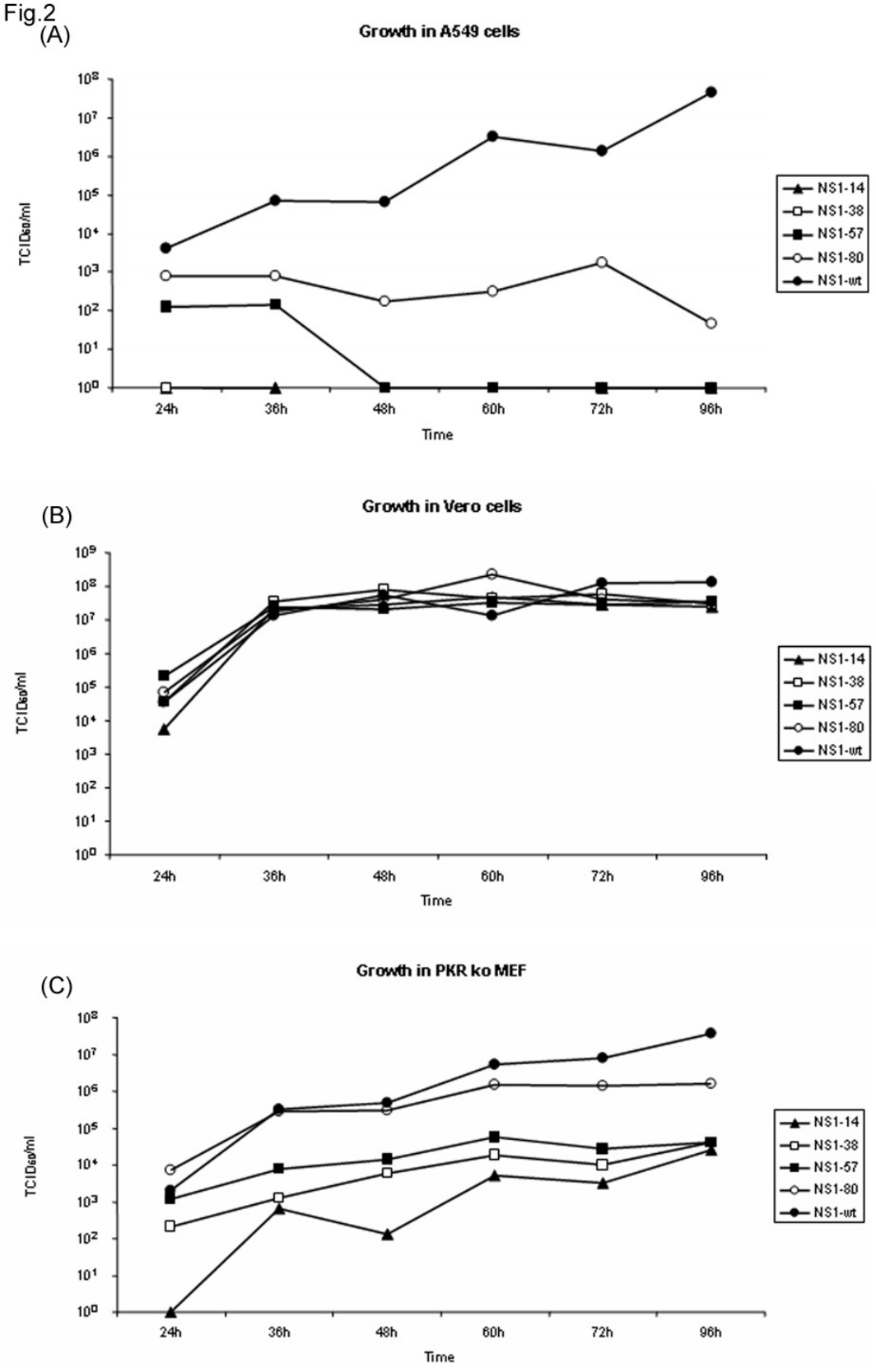


Figure 2: Growth properties of wt influenza B virus and NS1 truncation mutants in different cell lines. Confluent monolayers of (A) A549 cells, (B) Vero cells and (C) PKR knock out MEFs were infected with indicated viruses at an MOI of 0.01 and incubated at 33°C. At different time points, supernatants were harvested and the infectious titer was determined by $TCID_{50}$/ml. All values below the detection limit of $1*10^2$ $TCID_{50}$/ml were considered to be 10^0.

Influenza B NS1 truncation mutants induce antiviral and pro-inflammatory cytokines in macrophages and human nasal epithelial cells. It is well known that wt influenza viruses are able to antagonize type 1 IFN response as well as cytokine release from infected cells in various cell types (Dauber et al., 2004, Dauber et al., 2006, Egorov et al., 1998, Garcia-Sastre et al., 1998, Stasakova et al., 2005). In order to demonstrate the influence of influenza B virus NS1 protein on cytokine regulation we evaluated the potential of the NS1 truncation mutants NS1-14, NS1-38, NS1-80 and NS1-wt virus to induce IFN-α or IFN-β and major pro-inflammatory cytokines (TNF-α, IL-6 and IL1β) in 7-day-old human macrophages and primary nasal epithelial cells, respectively. The NS1-wt virus was fully competent to inhibit the release of IFN-α/β, TNF-α, IL-1 β and IL-6 whereas all NS1-truncated viruses induced markedly higher levels of indicated cytokines in both, macrophages and nasal epithelial cells (see Fig. 3). Although the NS1-80 mutant virus showed intermediate growth capacity in interferon competent cells, it seems that the first 80 aa of NS1 are not sufficient to block the activation of IFN and other pro inflammatory cytokines. Our data imply that carboxy-terminal deletions of the NS1 protein of influenza B viruses are associated with the loss of functions responsible for inhibiting pro-inflammatory and antiviral cytokine production in human macrophages and nasal epithelial cells.

Fig. 3

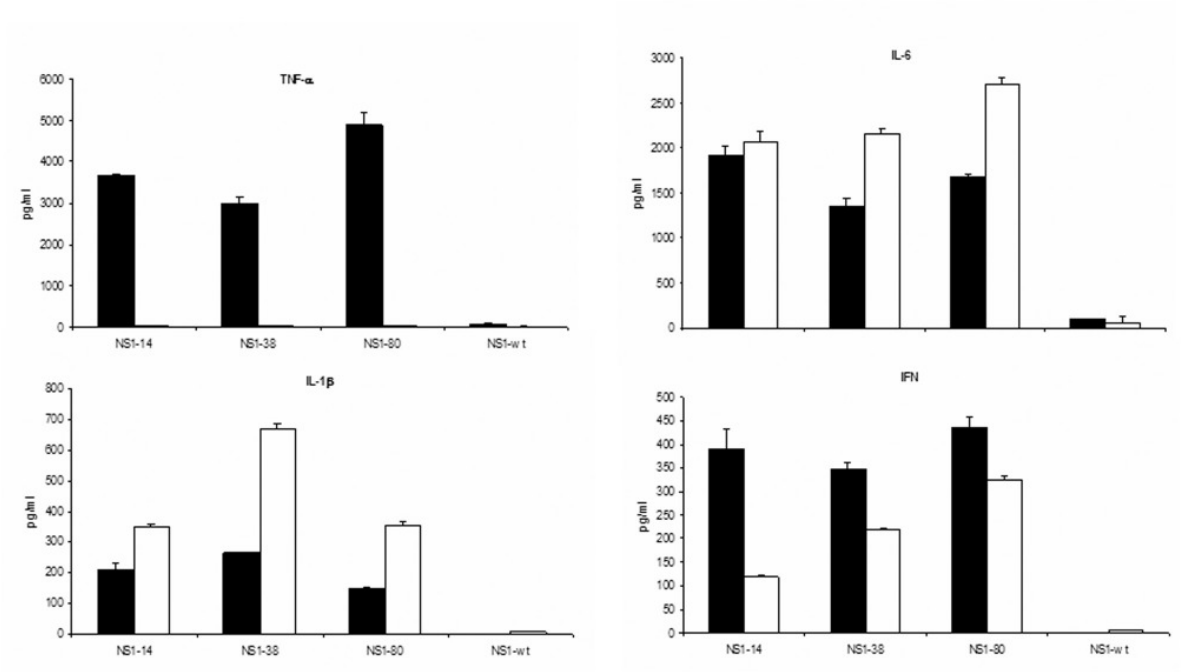


Figure 3: Cytokine release in human macrophages and HNEC infected with wt influenza B virus or NS1 truncation mutants. Human macrophages (black bars) and HNEC (white bars) were infected at an MOI of 2 with indicated viruses. Supernatants from infected cells were harvested 24h p.i. and assayed for TNF-α, IL-1β, IL-6 and IFN-α for macrophages and IFN-β for HNEC, respectively. One representative of three independent experiments is presented as a mean of two measurements +/- SEM. Mock value is subtracted.

Influenza B NS1 truncation mutants are replication-deficient in mice. Due to the attenuated replication pattern in A549 cells we used a mouse model to examine whether the same attenuating effect was observable in vivo. 6 to 8-week-old female BALB/c mice were infected i.n. with 5 x 10^5 $TCID_{50}$/mouse with either NS1 truncation or wt virus. Viral titers in lungs and nasal turbinates of mice were measured by $TCID_{50}$/ml of a 10% tissue homogenate in Vero cells 3 days p.i. The geometric mean titers are shown in Table 1. The influenza B NS1-wt virus was replicating to a titer of 3.83 x 10^4 $TCID_{50}$/ml of 10% tissue homogenate in lung tissue and 6.29 x 10^3 $TCID_{50}$/ml in nasal turbinates. All NS1-truncated viruses failed to be re-isolated from lungs and nasal tissue, which indicates a replication-deficient phenotype.

TABLE 1: Replication of wt influenza B virus or NS1 truncation mutants in mice

Virus	$TCID_{50}$/ml	
	Lung	Nasal turbinates
NS1-14	n.d.*	n.d.
NS1-38	n.d.	n.d.
NS1-57	n.d.	n.d.
NS1-80	n.d.	n.d.
NS1-wt	$3{,}83 \times 10^4$	$6{,}29 \times 10^3$
Control	n.d.	n.d.

* not detectable

Influenza B NS1 truncation mutants induce virus specific IgG response in mice. Subsequently, we investigated whether the replication-deficient NS1 truncation mutants induce a humoral immune response in mice. Animals immunized with any of the NS1 mutant viruses showed substantial virus-specific serum antibody levels even after one single i.n. immunization as determined by serum ELISA 29 days post immunization (Fig. 4). Differently to influenza B wt virus, a tendency to polarize the immune response towards Th1 was detected for the NS1 truncation mutants, reflected by a predominance of IgG2a antibodies over IgG1. Serum of non immunized control mice did not yield in any significant titer and did not show any polarization effect.

Fig. 4

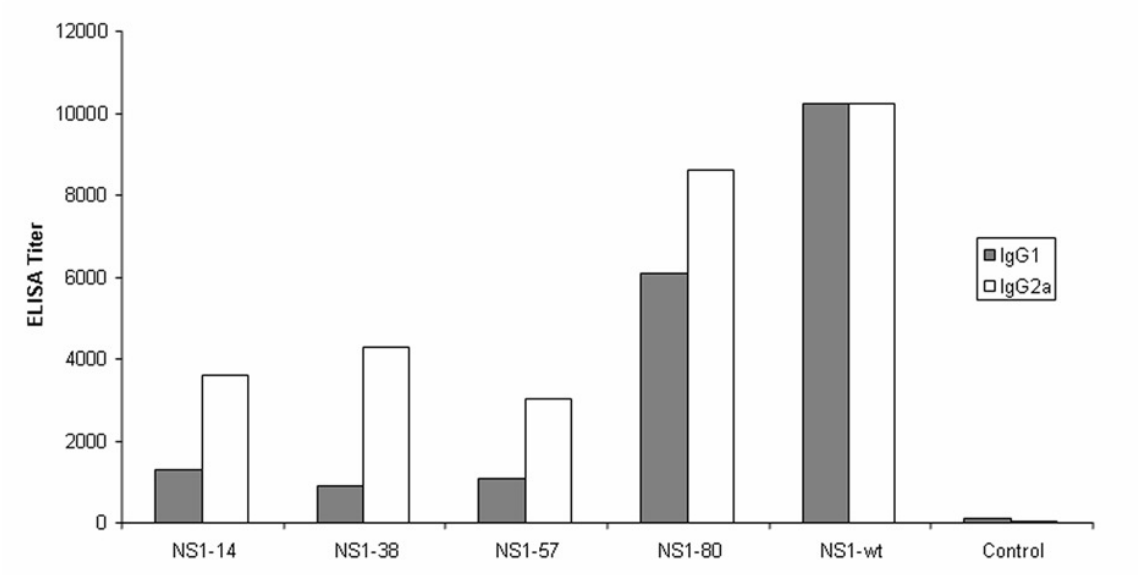


Figure 4: Detection of virus specific IgG1/IgG2a in serum of primed mice. BALB/c Mice were immunized i.n. with indicated viruses ($5*10^5$ $TCID_{50}$/mouse) or medium as a control. Serum samples were obtained 29 days after immunization. Virus specific IgG1 and IgG2a geometric mean titers were determined by ELISA and presented as one representative of two independent experiments.

Mice are protected against wt virus challenge after one single immunization with NS1 truncation mutants. To investigate whether a single, i.n. immunization of the replication-deficient NS1 truncation mutants induces protective immunity, mice were challenged with 5×10^5 $TCID_{50}$/mouse of homologous influenza NS1-wt 32 days post immunization. The challenge virus was derived from a human isolate and did therefore not induce any symptoms such as body weight loss or lethality in mice (data not shown). Mice were sacrificed 3 days post challenge and viral titers were determined in lungs and reported as geometrical mean titers (GMT) $TCID_{50}$/ml of a 10% tissue homogenate. Upon challenge with NS1-wt virus, none of the naïve mice was protected against infection, as indicated by viral loads of 2.47×10^4 $TCID_{50}$/ml in lung tissue. In contrast all mice immunized either with NS1-wt virus or with any of the NS1-truncated viruses were completely protected as demonstrated by the absence of detectable challenge viruses in their lungs (Table 2).

TABLE 2: Protection of mice immunized with influenza B truncation mutants against wt influenza B challenge

	Lung	
Virus for immunization	**$TCID_{50}$/ml**	**No. protected[§]/ total no.**
NS1-14	n.d.[*]	4/4
NS1-38	n.d.	4/4
NS1-57	n.d.	4/4
NS1-80	n.d.	4/4
NS1-wt	n.d.	3/3
Control	2.47×10^4	0/4

[*] not detectable

[§] Mice with a titer below the detection limit of $1x10^2$ $TCID_{50}$/ml are considered as protected.

6.5. DISCUSSION

Seasonal influenza vaccines need to protect against circulating type A and type B influenza viruses. The purpose of this study was to develop contemporary, genetically defined and attenuated influenza B vaccine strains for production in certified Vero cells, implying that rescued strains are suitable for i.n. immunization of humans. It was previously shown that influenza A viruses with carboxy-terminally truncated NS1 proteins of different lengths are attenuated in such IFN-competent systems as MDCK cells and mice. Their attenuation was depended on the length of their NS1 proteins but they were able to replicate to high titers in IFN-incompetent Vero cells (Egorov et al., 1998). This directly proved the role of A/NS1 in IFN antagonism. In a similar approach we developed a reverse genetic system employing contemporary influenza B virus adapted to high growth in Vero cells and generated a set of recombinant influenza B viruses with C-terminal deletions in their NS1 protein. The resulting viruses comprise NS1 proteins ranging from 14 to 80 aa, resulting in a loss of the complete functional RBD (Wang & Krug, 1996).

Our data show that the generated influenza B mutant viruses lacking a functional NS1 protein are capable to grow in IFN-deficient Vero cells and are highly attenuated in IFN-competent A549 cells, which is in accordance with data found for influenza A virus. This demonstrates that even though the influenza A and B virus NS1 proteins share only limited sequence homology and differ considerably in their regulatory functions, their major function of antagonizing the host's innate immune system is similar.

We show that the deletion mutant NS1-80 replicates to high titers in PKR ko MEFs but is attenuated in A549 cells. This indicates that PKR is one major viral growth restricting factor. This finding is in accordance with the data published by Dauber et al., who demonstrated that influenza B mutants with impaired RNA-binding capacity cannot block the activation of PKR and are therefore highly attenuated in IFN-competent cells (Dauber et al., 2006). Hence, the genetic inactivation of PKR is sufficient to largely rescue efficient replication of the NS1-80 virus. In contrast, deletion mutants shorter than 80 aa were highly attenuated in PKR ko MEFs. We

hypothesize that antiviral mechanisms other than PKR might be involved and that the N-terminal 80 aa of NS1 are sufficient to antagonize them. Mx and ISG15 could be excluded from the other IFN-induced antiviral proteins known to interact with influenza replication, because the former is not expressed in cells derived from most laboratory mice strains and the latter one is expected not to be recognized by B/NS1 protein of 80 aa (Yuan & Krug, 2001). Whether activation of 2',5'-OAS or other antiviral proteins can be blocked by the partial function of the N-terminal 80 aa of B/NS1 remains to be investigated.

We were able to rescue an influenza B virus expressing an NS1 protein as short as 14 amino acids, which we assume to have no function. However, we failed to generate an influenza B virus completely lacking the NS1 open reading frame, in which the NS2/NEP is expressed from a monocistronic and not from a spliced mRNA. Dauber et al. generated a ΔNS1 influenza B/Lee/40 virus (ΔNS1-B), which did not grow in 11-day-old embryonated eggs but could be propagated in 6-day-old eggs that have a premature immune system (Dauber et al., 2004). It is not clear yet why ΔNS1-B did not replicate in IFN-deficient Vero cells, while the analogous influenza A ΔNS1 virus did. One reason why the influenza B NS1-14 virus but not ΔNS1-B is able to replicate in Vero cells may be the lack of a natural splice site in ΔNS1-B, potentially leading to overexpression of NS2/NEP mRNA and resulting in a disregulation of nuclear export function or in a disparity in the cellular processing of spliced to unspliced protein (Reed & Cheng, 2005). We speculate that this change in the expression strategy of NS2/NEP could be compensated by other viral genes and that it might be possible to adapt a ΔNS1-B virus to replicate in Vero cells.

It was shown previously that influenza A mutant viruses lacking the NS1 gene or expressing a NS1 protein with an impaired RBD and dimerization domain induce 10-50 times higher levels of pro-inflammatory cytokines in infected macrophages as compared to wt virus (Stasakova et al., 2005). For type B viruses the amino acids downstream of NS1 position 104 were found essential for the control of hyper-induction of IFN-α/β in a continuous epithelial cell line (Dauber et al., 2006). The current study highlights the general importance of the B/NS1 protein in downregulating

pro-inflammatory and antiviral cytokines as shown by higher levels of IFN-α/β, TNF-α, IL-6 and IL1-β of the influenza B NS1 truncation mutants as compared to wt virus in macrophages and primary human nasal epithelial cells.

This report is the first description of a targeted approach to generate live influenza B vaccine strains whose attenuation mechanism is based on the partial deletion of the NS1 protein. The generated influenza B NS1 mutant viruses can be used for further clinical development of an i.n. administered live attenuated vaccine. They offer the advantage of being completely replication-deficient and not provoking any shedding. Immunized animals were completely protected from wt virus challenge in mouse lungs irrespectively of the length of the remaining NS1.

We could show that mice receiving one single dose of replication-deficient virus induced anti-influenza IgG titers and had the tendency to polarize the immune system towards Th1, which is considered the best correlate of protectivity against viral infection (Proietti et al., 2002, Tovey et al., 2008). Due to the lack of a functional NS1 protein, an increased local production of cytokines and chemokines potentially stimulates the humoral and cellular arm of the immune system. Because most inactivated vaccines are dependent on an adjuvant for stimulating such a response, we propose to call this phenomenon "self adjuvanting effect".

Our data emphasize that IFN-sensitive influenza B viruses with truncations in their NS1 gene can be efficiently produced in Vero cells and are immunogenic in animals. This presents a new perspective for further vaccine development strategies.

7. Development of A live-attenuated influenza B ΔNS1 intranasal vaccine candidate

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submitted for publication in J Vaccine

7.1. ABSTRACT

We discovered a unique, single amino acid mutation in the influenza B M1 protein promoting viral growth of NS1 truncation mutants in Vero cells. Due to this mutation, we were able to generate an influenza B virus lacking the complete NS1 open reading frame (ΔNS1-B virus) by reverse genetics, which was growing to titers of 8 $\log_{10}TCID_{50}$/ml in a Vero cell culture-based micro-carrier fermenter. The ΔNS1-B vaccine candidate was attenuated in IFN-competent hosts such as human alveolar epithelial cells (A549) similar to influenza A ΔNS1 viruses. In ferrets, the ΔNS1-B virus was replication-deficient and did not provoke any clinical symptoms. Importantly, a single intranasal immunization of ferrets at a dose as low as 6 $\log_{10}$ $TCID_{50}$/animal induced a significant HAI response and provided protection against challenge with wild-type influenza B virus. So far, the lack of a ΔNS1-B virus component growing to high titers in cell culture has been limiting the possibility to formulate a trivalent vaccine based on deletion of the NS1 gene. Our study closes this gap and paves the way for the clinical evaluation of a seasonal, trivalent, live replication-deficient ΔNS1 intranasal influenza vaccine.

7.2. INTRODUCTION

Influenza A and B viruses cause severe respiratory illness and have a tremendous social and economic impact. Responsible for thousands of deaths and hospitalizations per year in Europe alone, influenza viruses continue to be a major health concern (EISS, 2006). Whereas influenza A viruses have a high epidemiological impact, influenza B viruses are more associated with complications like Reye's syndrome and encephalitis in children (Lin et al., 2006, McCullers et al., 1999). Although antiviral drugs have already been licensed for human application, vaccination remains the primary strategy for preventing and controlling influenza.

Current influenza virus vaccines consist of three components, namely the influenza B component, influenza A H1N1 and H3N2. Due to antigenic drift the WHO recommends the strains to be included into the vaccine formulation semi-annually. For 2008/2009, all three components of the trivalent vaccine were changed to influenza A/Brisbane/53/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Florida/4/2006 for the northern hemisphere.

At present, two types of licensed influenza vaccines are available, inactivated (TIV) and live cold-adapted (*ca*) attenuated influenza vaccines (LAIV). Despite comparable efficacies of both TIVs and LAIVs (Belshe et al., 2008, Fleming et al., 2006, Treanor et al., 1999), LAIVs have the advantage of needle-free administration via the nasal mucosa and hence provoke a different spectrum of the immune response. While TIVs induce overall higher serum antibody titers, LAIVs elicit specific mucosal IgA antibodies at the site of virus entry, thereby helping to prevent infection before viral replication can occur (Doherty et al., 2006, Nichol, 2001, Nichol & Treanor, 2006). It is reported that LAIVs induce a longer lasting, broader and more protective immune response and the involvement of virus-specific T-cells and the induction of secretory IgA antibodies in the nasal mucosa suggests better cross-protection against antigenic drift variants (Belshe et al., 2000, Beyer et al., 2002, Cox et al., 2004, Ferko et al., 2004, Nichol & Treanor, 2006, Tamura et al., 2005). Although LAIVs are considered to be sufficiently safe, they clearly have the disadvantage

of viral replication in the upper respiratory tract resulting in shedding from vacinees. Particularly cold-adapted influenza B virus was shown to replicate up to 21 days after immunization in children(Vesikari et al., 2006b).

Alternative live attenuated vaccine approaches for influenza viruses have been developed, for instance, by altering the viral IFN antagonist, i.e. the NS1 protein, by reverse genetics (Egorov et al., 1998, Garcia-Sastre et al., 1998). Influenza A viruses with impaired NS1 function are not able to counteract the host's innate immune response (Hale et al., 2008). It was demonstrated that influenza A viruses with large C-terminal truncations in the NS1 protein are attenuated and induce protection in animals (Ferko et al., 2004, Kochs et al., 2007, Quinlivan et al., 2005, Solorzano et al., 2005). An influenza A virus devoid of the NS1 protein (ΔNS1-A) was found to be completely replication deficient in animals and induces high amounts of pro-inflammatory cytokines, associated with activation of antigen presenting dendritic cells as well as virus specific B- and T-cell mediated immune responses (Egorov et al., 1998, Ferko et al., 2004, Talon et al., 2000b). Recently, we performed a first-in-man study with a ΔNS1-A (H1N1) vaccine virus where the replication-deficient phenotype and high immunogenicity in humans was confirmed (Manuscript in preparation).

Until now a ΔNS1 influenza B virus with high growth potential in tissue culture was not available. In this study, we discovered an adaptive mutation in the M1 protein enabling replication of an influenza B virus in which the NS1 ORF was completely deleted (ΔNS1-B) to high titers of 8 $\log_{10}$ $TCID_{50}$/ml in a Vero cell culture-based micro-carrier fermenter. At the same time the virus was replication-deficient and apathogenic in animals. We demonstrate that ferrets immunized once intranasally with the ΔNS1-B vaccine candidate at a dose of 6 $\log_{10}$ $TCID_{50}$/animal elicited a systemic antibody response and were protected against challenge with wild-type virus after one single intranasal administration.

7.3. MATERIALS AND METHODS

7.3.1. Cells, viruses and viral infections

Vero cells (ATCC CCL-81) were used for transfection experiments, propagating viruses and virus titrations. Vero cells were adapted to and cultivated in serum-free OptiPro media (Invitrogen) with 4mM L-glutamine (Gibco). A549 cells (ATCC CCL-185) were cultivated in Dulbecco-modified Eagle medium (DMEM; Gibco) and used for growth curves of rescued viruses. Influenza B/Vienna/23/07 virus subtyped as influenza B/Florida/04/06, B/Vienna/33/06 subtyped as B/Malaysia/2506/04 were provided by M. Redlberger (Institute of Virology, Vienna, Austria) and B/Thüringen/02/06 virus subtyped as influenza B/Jiangsu/10/03 was obtained from B. Schweiger (German National Reference Center for Influenza; Robert Koch Institute, Berlin, Germany). For easier distinction, viruses were named according to the subtype of their HA and NA genes as B/Florida, B/Malaysia and B/Jiangsu, respectively. Influenza A/Vienna/28/06 virus subtyped as influenza H3N2 A/Wisconsin/67/05 and A/Vienna/414/07 subtyped as influenza H1N1 A/Brisbane/59/07 were provided by M. Redlberger (Institute of Virology, Vienna, Austria). The IVR-116 vaccine strain, a reassortant of influenza A/New Caledonia/20/99 and A/Puerto Rico/8/34 (PR8) viruses, was obtained by the WHO. All viruses were adapted to grow on serum-free Vero cells. To propagate viruses and to determine viral replication, indicated cells were washed with phosphate-buffered saline (PBS) and incubated with virus at the indicated multiplicities of infection (MOI) at room temperature for 30 minutes. Inoculum was removed, serum-free OptiPro media containing 5µg/ml Trypsin (Sigma) and 250ng/ml Amphotericin B (Brystol Meyers) was added and cells were incubated at 33°C for influenza B or 37° for influenza A viruses and 5% CO_2. After the indicated time points, the supernatant of infected cells was analyzed for viral titer by $TCID_{50}$.

7.3.2. Generation of NS1-truncated viruses

HA and NA genes of B/Florida virus and the remaining 6 genes of B/Jiangsu or all 8 genes of B/Malaysia including non-coding regions were sequenced and each cloned into vector phW2006, a synthetically produced vector similar to pHW2000 (Hoffmann et al., 2002), generated by GENEART (Regensburg, Germany). The vector contains PolI/PolII promoter and terminator sequences for the bicistronic expression of influenza genes. To generate the NS1 truncation mutants, the plasmid encoding the NS gene was modified by site-directed mutagenesis (Stratagene) to express an NS1 protein of 38 and 80 aa, respectively. Translation was terminated by two consecutive in-frame stop codons. The cDNA downstream of the stop codon cassette up to the splicing signal of NS2/NEP (position 634nt) was deleted by inverse PCR. Inverse PCR was performed to obtain a plasmid coding for an NS gene segment, in which the complete open reading frame of NS1 is deleted (nt position 78-731). A mutation in the plasmid encoding the M gene at nt position 281 (A-G) resulting in a change at amino acid position 86 of the M1 protein from valine to methionine was introduced in a similar way. All viruses were rescued by transfection on Vero cells as described previously (in press). Viruses used for growth curves and animal studies containing HA and NA of B/Florida and the remaining genes of B/Jiangsu comprising the M1-M86V mutation were named according to the size of the NS1 protein, i.e. ΔNS1-B and NS1-wt.

For the generation of the influenza H1N1 and H3N2 vaccine strains, the NS1 open reading frame of influenza A viruses was deleted in a similar way (Garcia-Sastre et al., 1998). Rescued viruses containing HA and NA A/Vienna/414/07 or from A/Vienna/28/06, the ΔNS1 gene and the other genes from IVR116 were named as ΔNS1-H1N1 and ΔNS1- H3N2, respectively. The expected sequences of HA, NA and NS genes of rescued viruses were confirmed by nucleotide sequence analysis and, for NS, additionally by analyzing amplified RT-PCR products.

7.3.3. Production of influenza virus in Vero cell culture-based micro-carrier fermenter

Growth of influenza virus in Vero cells was as described elsewhere (Kistner et al., 1998). Briefly, a single aliquot of the fully characterized Vero production cell bank was expanded through successive passages to the required volume in serum-free DMEM/Ham's F12 medium. 15 L-scale fermenters containing microcarrier cultures were infected with an aliquot of the ΔNS1-B influenza virus at an MOI of 0.005. Incubation was carried out at 34°C for 3-4 days using trypsin at a concentration of 20 mU/ml. Supernatant virus was harvested, clarified and analyzed for HA and $TCID_{50}$.

7.3.4. Immunization and challenge of ferrets

Ferrets were housed individually at 15°C–21ºC, a relative humidity of 30–70% and a 12:12 light:dark cycle. Food (Catmix complete, Dibaq, Czech Republic) and water were provided *ad libitum*. Four to five animals per group of 5 to 8-months-old male and female ferrets (*Mustela putorius furo)* were intranasally (i.n.) infected under anaesthesia (Ketamin 20mg/kg + Medetomidine 0.04mg/kg, i.m.) at 1 x 10^6 $TCID_{50}$/ferret with either monovalent influenza B virus ΔNS1-B vaccine or with 1 x 10^6 $TCID_{50}$/ferret of each strain ΔNS1-B, ΔNS1-H1N1 and ΔNS1-H3N2 (trivalent formulation) or PBS (control group). Ferrets were monitored daily for clinical signs such as sneezing and nasal discharge and body temperature and body weight were recorded. Nasal washes were taken from all animals on days 3 and 5 for analysis of viral replication in tissue culture. Blood was collected 4 days prior and 29 days after immunization, sera were prepared and further used for the hemagglutination inhibition assays. On day 37 post immunization animals were challenged with influenza B NS1-wt strain (1 x 10^6 $TCID_{50}$/ferret) and nasal washes were taken on days 3 and 5 post challenge to determine the viral load as described above. This animal study was approved by the Animal Care and Use Committee (IACUC) of BioTest Ltd. and the Committee for Animal

Protection of the Ministry of Industry and Trade of the Czech Republic (35/2007).

7.3.5. Hemagglutination inhibition (HAI) assay

Serial twofold dilutions of ferret sera pre-treated with receptor-destroying enzyme (RDE) were prepared in a 96-well micro titer plate and 25µl containing 4 HA units of homologous antigen was added to each well. After an incubation period of 30 minutes at room temperature, 50µl of chicken erythrocytes (0.5%, derived from adult chicken blood) were added. Results were recorded after incubation at 4°C for 60 minutes. The HAI titration end point was determined by the highest dilution of antiserum that still inhibits complete hemagglutination of RBCs. The presented value is the reciprocal of the last serum dilution inhibiting virus hemagglutination. A titer of 1:8 was considered to be negative. Values from the monovalent and trivalent ΔNS1-B groups were each compared to the PBS group by Mann-Whitney U test.

7.4. RESULTS

Generation of influenza B NS1 mutant viruses on Vero cells

Influenza B NS1 mutants were generated by reverse genetics on Vero cells using 8 plasmid rescue system as described previously (Hoffmann et al., 2002, Kittel et al., 2005). Initial rescue experiments with B/Malaysia NS1-80 and NS1-wt virus showed clear cytopathic effects (CPE) three to four days post transfection whereas NS1-38 only showed weak CPE after 3 blind passages on Vero cells (data not shown). Higher growth of the NS1-38 virus was observed after further passaging on Vero cells suggesting that an adaptive mutation had occurred. Sequencing of the whole virus genome of NS1-38 revealed a single coding mutation in the M1 gene at nucleotide position 281, corresponding to an amino acid change at position 86 from methionine to valine (Fig. 1A). A sequence query showed that the M1-M86V mutation is a unique mutation not present in any other published viral M1 sequence (Fig. 1). Reintroduction of the M1-M86V mutation into the B/Malaysia backbone confirmed the effect of this mutation in boosting the viral growth of a NS1-38 mutant virus (data not shown).

Fig. 1

Vaccine, Wressnigg et. al

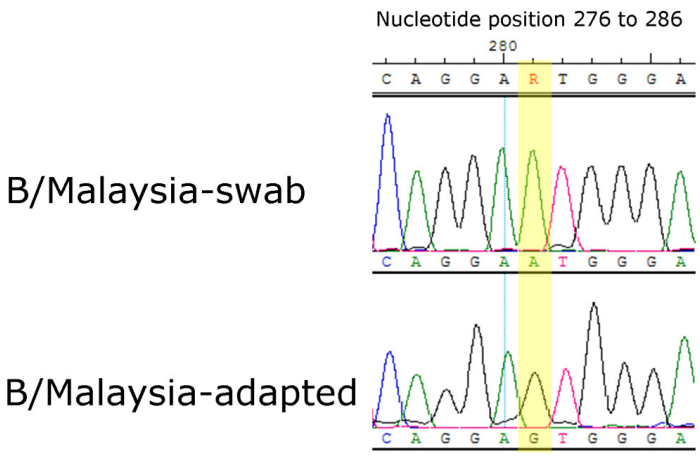

Figure 1: Sequencing diagramm of M genes of influenza B/Malaysia-swab and B/Malaysia-adapted virus and comparison with other influenza B viruses.

(A) A B/Malaysia virus containing an NS1 protein of 38aa was rescued on Vero cells, passaged and the M gene of the B/Malaysia-adapted was compared by sequencing to the parental wt strain. One nucleotide difference at position 281 resulting in an amino acid change at position 86 (M86V) was found.

To evaluate the impact of the M1-M86V mutation on other influenza B strains, we introduced this mutation into a different viral backbone. We created a reverse genetic rescue set based on the B/Jiangsu master strain containing HA and NA glycoproteins of B/Florida, the WHO recommendation for the year 2007/08. The M1-M86V substitution slightly improved the growth of NS1-wt virus when compared to the wt M gene construct (Fig. 2B). However, viral titers for the NS1-38 and NS1-80 mutants containing the M1-M86V mutation were approximately 4 or 2 logs higher than wt M1 analogs, respectively. Due to the M1-M86V mutation, we were able to rescue for the first time a ΔNS1-B virus reaching titers of almost 4 logs 5 days post transfection on Vero cells. These data demonstrate that the M1-M86V mutation compensates for a dysfunctional NS1 protein and is essential for rescuing a ΔNS1-B virus growing to high titers on Vero cells, in which the NS1 ORF is completely deleted.

Fig. 2

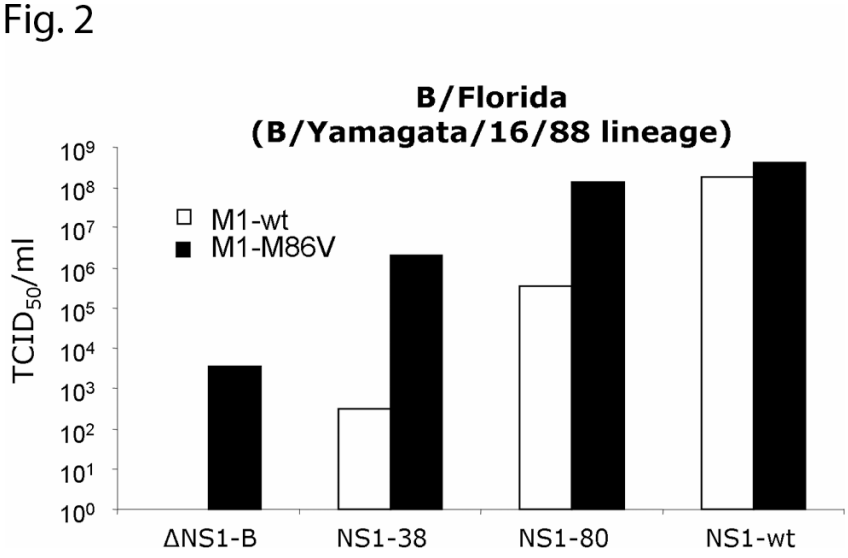

Figure 2: Viral titer of recombinant influenza B viruses containing wt or M86V M gene after transfection. Supernatant of indicated viruses was harvested 5 days post transfection containing the M gene as indicated. Titer was measured by $TCID^{50}$ on Vero cells.

Influenza ΔNS1-B virus is attenuated in A549 cells but replicates efficiently in a Vero cell culture-based micro-carrier fermenter

In order to demonstrate the interferon sensitive phenotype of ΔNS1-B virus, we examined its ability to grow on IFN-competent A549 and IFN-deficient Vero cells in comparison to the corresponding wt virus (Fig. 3). Both viruses showed comparable growth kinetics on Vero cells, reaching titers in the range of 10^7 to 10^8 $TCID_{50}$/ml. Replication of ΔNS1-B virus was completely restricted in IFN-competent A549 cells, which displayed no growth above the detection limit of 2 x 10^2 $TCID_{50}$/ml, while NS1-wt virus replicated to high titers of 3.15 x 10^8 $TCID_{50}$/ml. Mutant viruses NS1-80 and NS1-38 showed intermediate replication capacity (in press).

Fig 3a

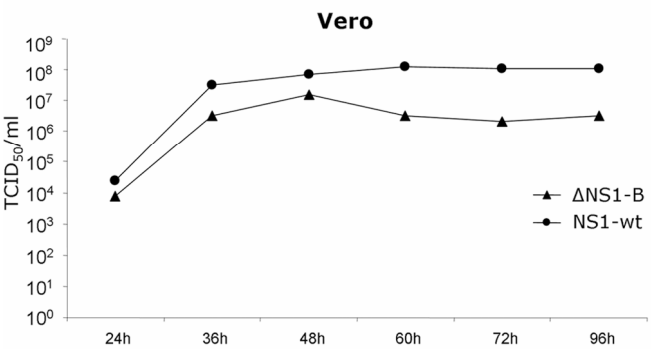

Fig. 3b

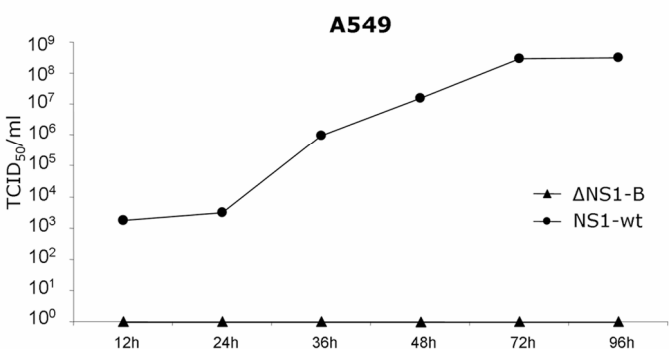

Figure 3: Growth properties of influenza B viruses in Vero and A549 cell lines. Confluent monolayers of (A) Vero cells and (B) A549 cells were infected with indicated viruses at an MOI of 0.01 and incubated at 33°C. At different time points, supernatants were harvested and the infectious titer was determined by $TCID_{50}$. All values below the detection limit of 1 x 10^2 $TCID_{50}$ were considered to be 10^0.

Furthermore we could demonstrate that the ΔNS1-B vaccine candidate could be efficiently produced in a Vero cell culture-based micro-carrier fermenter (Table 1). In three independent 15L fermenter runs the ΔNS1-B virus yielded HA titers of 48 and infectious titers were ranging from 7.80 +/- 0.19 to 8.00 +/- 0.25 $\log_{10}$ $TCID_{50}$/ml. These titers were equal or even higher than the titers obtained in monolayer Vero cell culture. We demonstrate that the ΔNS1-B virus can be successfully produced in Vero cell culture-based micro-carrier fermenter reaching high titers of 8 $\log_{10}$.

TABLE 1: Hemagglutination and $TCID_{50}$ titers of ΔNS1-B vaccine in three independent GMP fermentations

	Fermentation	
Clarified GMP harvest	**$Log_{10}TCID_{50}$/ml**	**HAU[a]/50µl**
Batch 1	8.00 +/- 0.25	48
Batch 2	7.80 +/- 0.19	48
Batch 3	7.95 +/- 0.33	48

[a]Hemagglutinating Units

Influenza ΔNS1-B vaccine is replication-deficient and safe in ferrets

In view of the attenuated replication pattern in A549 cells, we used the ferret model to examine whether the same attenuating effect was observable in vivo. 5 to 8-months-old male and female ferrets (*Mustela putorius furo)* were infected i.n. with ΔNS1-B virus at a dosage of 1 x 10^6 $TCID_{50}$/ferret. Viral titers in the nasal washes of animals were measured by $TCID_{50}$/ml on Vero cells 2 days p.i. No virus could be recovered for ΔNS1-B virus indicating a replication-deficient phenotype without any vaccine virus shedding (data not shown). Moreover all animals were monitored daily for clinical symptoms and activity status (inactivity/lethargy). No respiratory symptoms such as coughing, nasal discharge, sneezing and dyspnoe could be observed for the vaccine group, nor were any neurological symptoms such as ataxia, limb paresis, torticollis or abnormal behavior detectable. No changes in body weight and body temperature were recorded (data not shown). These data indicate that the ΔNS1-B vaccine candidate is safe.

Influenza ΔNS1-B vaccine is immunogenic in ferrets

Subsequently, we investigated whether the ΔNS1-B vaccine virus induces a systemic antibody immune response in ferrets. Animals were immunized with the monovalent vaccine containing 1 x 10^6 $TCID_{50}$/ferret of ΔNS1-B virus. In order to study the interference with influenza A ΔNS1 mutant viruses in a trivalent formulation, another group of animals was

vaccinated with the trivalent vaccine containing 1 x 10^6 $TCID_{50}$/ferret of ΔNS1-H1N1, ΔNS1-H3N2 and ΔNS1-B. Although higher doses would have been technically feasible since the ΔNS1-B vaccine strain was replicating to titers of 1 x 10^8 $TCID_{50}$/ml, we chose a rather low immunization dose to challenge our vaccine concept. Immune response against the influenza B component was analysed by hemagglutination inhibition assay (HAI) 29 days after immunization (Fig. 4). Ferrets receiving the monovalent ΔNS1-B vaccine showed significant hemagglutination-inhibition titers after immunization when compared to the PBS group, ranging from 32 to 64 ($p<0.05$). In the trivalent group the HAI response to the influenza B component was in the range of 8 to 16 ($p<0.05$ compared to the PBS group). Thus, to some extent an interference effect was detectable for the influenza B component (Fig. 4). However, this interference on the immunogenicity level did not affect protection against wild-type challenge (Fig 5).

Fig. 4

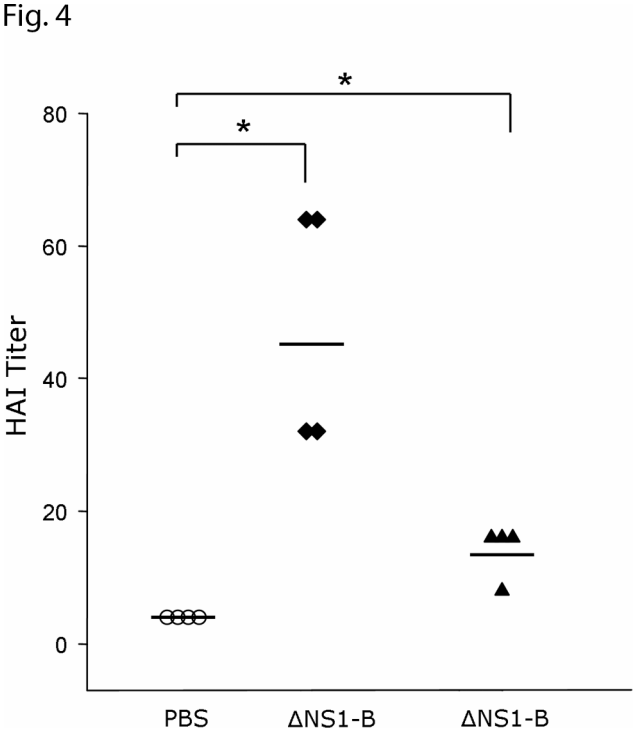

Figure 4: Hemagglutination inhibition assay of monovalent and trivalent vaccine. Sera from ferrets immunized with 1 x 106 TCID50/ferret of either monovalent ΔNS1-B virus ( ) or trivalent formulation ( ) were analyzed 29 days after immunization for hemagglutination inhibition titers against influenza B wt virus and compared to PBS group ( ) (*P< 0.05).

Influenza ΔNS1-B vaccine protects ferrets against wild-type virus challenge after one single immunization

We investigated whether one single i.n. immunization at a dose of 6 log_{10} $TCID_{50}$/ferret with the ΔNS1-B virus induces protective immunity. Ferrets were vaccinated with either monovalent or trivalent ΔNS1-B formulation

and were challenged with 1 x 10^6 $TCID_{50}$/ferret of homologous influenza B/Florida wt virus. Viral recovery of the nasal washings was chosen as the assessment criteria for protection since fever was not induced by challenge with wt virus. Samples were taken from ferrets on days 3 and 5 post challenge and viral titers were determined by $TCID_{50}$ assay.

In all five naïve control ferrets the challenge virus was detectable on day 3, as indicated by viral loads in the range of 3.59 x 10^1 to 1.47 x 10^3 $TCID_{50}$/ml in the nasal washes (Fig. 5). In contrast, three out of four ferrets immunized with either the monovalent ΔNS1-B vaccine virus or the trivalent formulation were fully protected showing no detectable challenge virus in nasal samples. A similar trend was observed on day 5 post challenge (Fig. 5). This finding demonstrates the effectiveness of the vaccine applied as monovalent or trivalent ΔNS1 virus preparation in protecting ferrets against challenge with wild-type virus after one single i.n. immunization.

Fig. 5

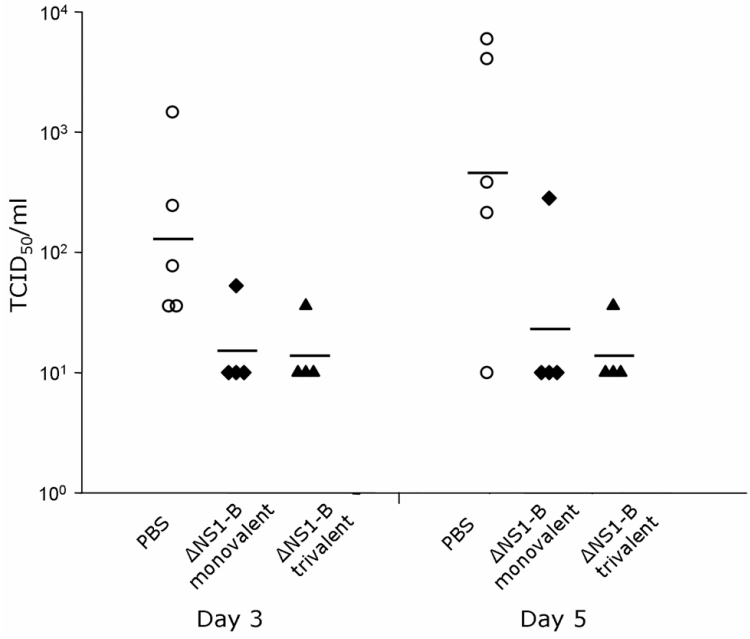

Figure 5: Viral recovery in nasal washes after challenge. Ferrets were i.n. immunized with 1 x 10^6 $TCID_{50}$/ferret of ΔNS1-B monovalent ( ) or trivalent ( ) and PBS ( ) and challenged 37 days post inoculation with influenza B wt virus (1 x 10^6 $TCID_{50}$/ferret). Viral replication was analyzed 3 and 5 days post challenge. Titers in the nasal washes were determined as $TCID_{50}$/ml and reported for every single ferret. Ferrets with a titer below the detection limit of 1 x 10^1 $TCID_{50}$/ml are considered as protected.

7.5. DISCUSSION

This is the first description of a contemporary, live attenuated influenza ΔNS1-B vaccine candidate strain whose attenuation mechanism is based on the deletion of the ORF of the NS1 protein and which is growing to high titers on Vero cells. Despite a replication deficient phenotype in ferrets, the monovalent ΔNS1-B and the trivalent vaccine formulation provoke a hemagglutination inhibition antibody response and protect animals from a homologous influenza virus challenge after one single intranasal immunization. The trivalent vaccine formulation additionally contains an influenza A ΔNS1-H1N1 and ΔNS1-H3N2 component. Importantly, while titers where reaching 8 $\log_{10}$ $TCID_{50}$/ml in a Vero cell culture-based micro-carrier fermenter, the immunogenic dose was as low as 6 $\log_{10}$ $TCID_{50}$/ferret.

When adapting an influenza B virus with a truncated NS1 protein on Vero cells, we discovered a unique mutation in the M1 (M1-M86V) protein, which is not present in any other influenza B virus sequences submitted to the Genbank. Reintroduction of this mutation into two independent viral backbones of representatives of the B/Yamagata/16/88 and B/Victoria/2/87 (data not shown) lineages resulted in increased rescue efficiency of viruses expressing C-terminal-truncated NS1 proteins. Only by inserting the M1 mutation we succeeded in generating a ΔNS1-B virus growing to high titers on Vero cells. Introducing the M1 mutation into the wt backbone resulted in slightly increased virus titers on Vero cells. Hence, the M1-M86V mutation might rather compensate for the loss of the functional NS1 protein than affecting Vero cell adaptation. The exact mechanism of the M1-M86V mutation remains to be further investigated.

Dauber et al. described the generation of a similar ΔNS1-B influenza virus, in which the ORF of NS1 is completely deleted and the NS2/NEP protein is expressed as a monocistronic mRNA. This ΔNS1-B virus in the backbone of the egg-adapted B/Lee/40 strain showed severe attenuation compared to NS1 wild-type virus on IFN-deficient Vero cells reaching a titer of 2 logs (Dauber et al., 2004). Hai et al. generated an influenza B

(B/Yamagata/16/88) mutant virus with a truncated NS1 protein of 16 amino acids length (described as deltaNS1) (Hai et al., 2008). This virus was also shown to be severely attenuated in growth in Vero and other cells by replicating to less than 4 $\log_{10}$. It was hypothesized that the NS1-B protein plays an essential role in promoting high-level replication of the influenza B virus in both IFN-competent and IFN-deficient cell lines. Since high replication of vaccine viruses is crucial for commercial vaccine production, high titers in cell culture are mandatory. Our data show that it is feasible to produce a ΔNS1-B vaccine strain on certified Vero cells as demonstrated by titers of more than 8 $\log_{10}$ in a 15L Vero cell based microcarrier fermentation and that the NS1 protein of influenza B viruses is not needed for efficient replication in Vero cells, analogous to influenza A ΔNS1-A viruses.

We believe that the advantages of a replication deficient ΔNS1-B vaccine to attenuated replicating vaccines are versatile. In our work we confirmed that the deletion of the NS1 ORF in the influenza B virus genome leads to a replication deficient phenotype in animals similar to influenza A viruses. It is controversially discussed whether lack of replication of the vaccine virus might be a limiting factor for induction of protective immune response. Despite their replication-deficiency, ΔNS1 viruses are competent to infect the host cell and initiate transcription and translation of viral proteins. We demonstrate that replication of an influenza B vaccine virus in the respiratory tract is not essential for the induction of HAI and protective immune response in ferrets. The immunogenicity and protective efficacy at the relatively low dose of 6 $\log_{10}$ $TCID_{50}$/ml of ΔNS1-B viruses could be explained by their enhanced capacity to induce a local cytokine response at the site of vaccine application (Ferko et al., 2004). This "self adjuvant" effect potentially boosts the immune response and thereby acts as a natural adjuvant.

Although intermediate NS1 truncation mutants appear to be better immunogenic than complete deletion mutants in the mouse model (Ferko et al., 2004, Hai et al., 2008), we did not observe significant differences in immunogenicity between intermediate and complete NS deletion mutants of influenza A vaccine candidates in the ferret or macaque model

(manuscript in preparation). Viruses with NS1 proteins of intermediate length are still able to at least partially replicate in the respiratory tract inducing viral shedding like LAIVs. It was shown that cold-adapted B vaccine strains could replicate in vaccinated children up to 21 days after immunization and that they could be transmitted from vaccine recipients to contact persons (Block et al., 2008, Dauber et al., 2006, Hai et al., 2008, King et al., 2000, Talon et al., 2000b, Vesikari et al., 2006a). Therefore, it is recommended that individuals vaccinated with LAIV should not be in contact with immunosuppressed persons for 7 days after vaccination (King et al., 2000).

Except for viral shedding, ΔNS1 vaccines share basic properties of LAIVs such as ease of intranasal administration, induction of a mucosal IgA immune response, initiation of a strong cytotoxic T-cell response and cross-protection (Ferko et al., 2004). Certainly the fast generation of seed viruses by reverse genetics and vaccine manufacturing in large quantities in tissue culture offer additional advantages over classical reassortants and production in eggs. Vero cells are an IFN-deficient, certified cell line suitable for vaccine production and promote growth of ΔNS1 to high titers. Our study underlines the feasibility of producing a live attenuated, trivalent influenza vaccine on Vero cells based on ΔNS1 technology.

8. Conclusion

Despite the continuous improvement and advanced technologic developments of influenza vaccines, influenza viruses continue to be important pathogens with worldwide prevalence responsible for thousands of deaths and hospitalizations annually.

Within this thesis several influenza B viruses with C-terminally truncated NS1 proteins of different length were generated employing reverse genetics on Vero cells to progress the development of live-attenauted vaccines. Recapitulating the results:

(1) The rescued truncation mutants containing the N-terminal 14, 38, 57 or 80aa of the NS1 protein were impaired in their ability to counteract IFN production, were able to grow to high titers in IFN-deficient Vero cells and are severely attenuated in IFN-competent hosts. (2) They induce pro-inflammatory antiviral cytokines early after infection. (3) A NS1-80 virus displayed high growth on PKR knock-out mouse embryonic-fibroblasts, whereas NS1-truncated mutants with a NS1 protein shorter than 80aa were highly attenuated. This finding indicates that other antiviral mechanisms besides PKR might be involved and that the first 80aa of the NS1 protein are sufficient to antagonize these. Whether activation of 2',5'-OAS or other antiviral proteins can be blocked by the partial function of the N-terminal 80 aa of B/NS1 remains to be elucidated. (4) Mice receiving a single intranasal immunization of the NS1-truncated viruses elicited an antibody response and were protected from wild-type virus challenge. (5) A complete ΔNS1-B virus was feasible to rescue due to the introduction of a single mutation in the M1 gene. (6) It was shown that despite the lack of a function IFN-antagonizing protein NS1 this influenza B virus was growing to high titers in Vero cells and at the same time was replication deficient in IFN-competent hosts such as A549 cells and ferrets. (7) A single intranasal immunization of ferrets at a dose as low as 6 $\log_{10}$ $TCID_{50}$/animal induced a significant HAI response and provided protection against challenge with wild-type influenza B virus.

In the scientific community it is controversially discussed whether a complete ΔNS1 virus or an intermediate mutant is more appropriate for application as a life vaccine. We believe that the advantages of a ΔNS1-B vaccine to intermediate NS1 mutant viruses are multifaceted. Viruses with NS1 proteins of intermediate length are still able to at least partially replicate in IFN-competent hosts (Dauber et al., 2006, Hai et al., 2008, Talon et al., 2000b). Some believes are that replication-competence in the host is a benefit in contrast to replication-deficiency (Hale et al., 2008), neglecting the fact that viral shedding like LAIVs is induced. It was made evident that cold-adapted B vaccine strains could replicate in vaccinated children up to 21 days after immunization and that transmission from vaccine recipients to contact persons occurs (Block et al., 2008, King et al., 2000, Vesikari et al., 2006a). Furthermore Talon et al. investigated the properties of two spontaneous influenza B virus mutants expressing 110 and 89 amino acids of the NS1 protein, respectively. Both NS1 mutant viruses replicated in mouse lungs up to 3 days after infection (Talon et al., 2000b). Hai et al. generated influenza B NS1 truncation viruses consisting of 16, 80 and 110 amino acids, respectively, which replicated up to 3 logs in mice. In contrast to these two studies, we were neither able to isolate shedding virus from ferrets immunized with ΔNS1-B which contains a complete deletion of NS1 nor could we detect any sign of replication on IFN-competent cells such as A549. Despite their replication-deficiency, ΔNS1 viruses are competent to infect the host cell, initiate transcription and translation of viral proteins and induce HAI and a protective immune response in ferrets.

This study underlines the applicability of a ΔNS1-B virus as a live attenuated influenza vaccine and emphasizes the feasibility of producing a trivalent vaccine.

9. Abbreviations

DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethylsulfoxide
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
HA	Hemaglutinin
HAI	Hemagglutination Inhibition Assay
IgG	Immunglobulin G
IL	Interleukin
kDa	Kilo-Dalton
M	Matrix protein
NA	Neuraminidase
NCR	Non coding regions
NEP	Nuclear export protein
NES	Nuclear export signal
NLS	Nuclear localization signal
NP	Nucleoprotein
NS1	Non structural protein 1
ORF	Open reading frame
PA	Polymerase (acidic)
PB1	Polymerase (basic) 1
PB2	Polymerase (basic) 2
PBS	Phosphate-buffered Saline
PKR	Protein kinase R

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Academic Curriculum

Since 09 2004	**University of Vienna**, Dept. of Microbiology and Genetics, Vienna, Austria *External Doctoral Candidate* Thesis Committee: Prof. T. Muster and Prof. T. Decker (University of Vienna) Thesis Title: Influenza B NS1 truncation mutants: A live attenuated vaccine approach
09 2004 – 07 2006	**Mount Sinai School of Medicine**, Dept. of Microbiology, New York City, USA *Graduate Visiting Researcher* in the laboratory of Prof. P. Palese Development of new strategies for live attenuated influenza vaccines
09 2001 – 03 2004	**University of Vienna**, Dept. of Microbiology and Genetics, Vienna, Austria *Master Degree in Microbiology* Thesis Committee: Prof. T. Muster and Dr. A. Grassauer Thesis Title: Effects of PDTC on Respiratory Syncitial Virus and Parainfluenza Virus
09 1998 – 09 2001	**University of Vienna**, Dept. of Biology, Vienna, Austria *Propedeutic Exam* ("Erster Abschnitt") in Biology
08 1997– 08 1998	**University of Vienna**, Department of History, Vienna, Austria Studies of the History of Art
08 1997	**Kollegium Kalksburg**, Kalksburg, Austria *High School Degree* ("Matura") with majors in Biology, German, and English

Professional Experience

Since 08 2004	**Green Hills Biotechnology,** Influenza B Group, Vienna, Austria *Graduate Researcher* (On Leave 09 2004- 07 2006) Development of live attenuated influenza vaccines
03 2003 – 08 2004	**Green Hills Biotechnology,** Process Development Group, Vienna, Austria *Research Assistant* Parainfluenza Viruses
Summer 2003	**Boehringer-Ingelheim**, Vienna, Austria *Summer Intern* Research in the Department of Oncology under the direction of Dr. T. Voss

Summer 2002 **Ludwig-Boltzmann Institute**, Institute for Cytocine-Research, Vienna, Austria
Research Intern
Research in the area of Chronic Lymphocytic Leukemia under the direction of Prof. Schwarzmeier

Previous internships at GlaxoSmithKline, Siemens Automotive and the ORES Whale Research Program of the Canadian government

Conferences, Achievements and Presentations

- International Congress of Virology (IUMS), Istanbul, Turkey (2008)
- Third European Congress of Virology, Nuernberg, Germany (2007)
- "Options for the Control of Influenza" Conference, Toronto, Canada (2007)
- 22. Ernst Klenk Symposium "Neue Infektionskrankheiten", Cologne, Germany (2005)
- International Union of Microbiological Sciences Meetings, San Francisco, USA (2005)
- Scholarship by Mount Sinai School of Medicine and Green Hills Biotechnology (2004-2006; Full Board and Tuition)
- Life Science Austria Seminar Circle, Vienna, Austria (2004)
- The Annual Symposium of the Circle of Virology, Berlin, Germany (2003)

Publications

1. M. Shehata, J.D. Schwarzmeier, M. Hilgarth, S. Strommer, D. Richter, A.Hölbl, **N. Wressnigg**, R. Hubmann, M.Duechler.
Bone Marrow Fibroblasts Support Survival of B-CLL Cells: Involvement of PI-3K. *Journal of Interferon & Cytokine Research*, *2002*

2. M. Shehata, J. D. Schwarzmeier, M. Hilgarth, S. Strommer, A. Hölbl, **N. Wressnigg**, R.Hubmann, M. Duechler
Human Stromal Fibroblasts Inhibit Apoptosis of B-CLL Cells through a Mechanism Involving Phosphatidylinositol 3-Kinase Pathway. *Blood* , *2002*

3**. N. Wressnigg**, A. P. Shurygina, T. Wolff, M. Redlberger-Fritz, T. Popow-Kraupp, T. Muster, A. Egorov and C. Kittel
Influenza B mutant viruses with truncated NS1 proteins grow efficiently in Vero cells and are immunogenic in mice. *J Gen Virol 2009*

4. **N.Wressnigg**, D. Voss, T. Wolff, J. Romanova, T. Ruthsatz, I. Mayerhofer, M. Reiter, S.Nakowitsch, J. Humer, A. Morokutti, T. Muster, A. Egorov and C. Kittel
Development of a live-attenuated influenza B ΔNS1 intranasal vaccine candidate. *(submitted for publication)*

Patents

C. Kittel, **Nina Wressnigg**
A single amino acid change in the M1 protein of influenza B leads to high growth properties on Vero and enables the rescue of viruses expressing short NS1 fragments.
Patent disclosed

Skills and Languages

Languages Fluent in German and English, basic Indonesian
PC skills Excellent Ms Office, various software products with molecular biological background. (Pymol, DNAstar, GeneRunner ...), Endnote